Differential neuronal involvement in the cobalt epilepsy model

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The work reported in this thesis was executed entirely by myself with the exception of the experiments involving assay of noradrenaline metabolites which were performed jointly with Dr. I.A.M. Pullar.

ABSTRACT OF THESIS

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Title of Thesis Differential neuronal involvement in the cobalt epilepsy model.

Various parameters of the gabaminergic, nor-adrenergic and serotonergic c cortical innervation were examined during the initiation, development and eventual resolution of the chronic epileptic focus produced in the rat by cortical cobalt implant. These parameters included levels of neurotransmitter, metabolites, synthetic and catabolic enzymes, and E.E.G. correlates. Cobalt levels were assayed in various areas of the cobalt-implanted rat brain; general protein synthesis and certain aspects of the pharmacology of the serotonergic system in this model were also investigated.

A hypothesis is offered concerning the possible interactions between the anterior Raphe nuclei, as a cortical synchronising organ, and the electrical events in the cortex associated with the epileptic focus.

The study of the gabbaminergic system was confined to assaying gamma-amino butyrate transferase (GABAT), as glutamate decarboxylase (GAD) and GABA had already been examined in this model; the GABAT assay was intended as a link between the information already available and the new data concerning the nor-adrenergic (NAd) and serotonergic (5HT) systems. It was found that levels of this enzyme follow a very similar pattern to the thirty-day cycle as previously established in this model in the MRC Brain Metabolism Unit, falling during the first four days after implant and only returning to normal in primary and secondary focal areas at around day 30. A temporary peak of GABAT at day 9 was ascribed to the glial reaction that develops at this stage.

Tyrosine hydroxylase (TOH), mono-amine oxidase (MAO), and catechol-o-methyl transferase (COMT) in primary and secondary focal areas also fell to very low levels during the first few days after implant, but only returned to normal after 75 - 100 days. This time-course agreed with the metabolite data. Homovanillic acid (HVA) and 3-methoxy 4-hydroxy phenyl glycol (HMPG) levels were measured in parallel groups of rats. The changes in TOH were restricted to the NAd nerve-terminals, as no similar change could be detected in the cell bodies in locus coeruleus. COMT and MAO peaked shortly before GABAT, and were also ascribed to the gliosis; the HMPG peak that also developed at this stage was thought to be linked to the altered enzyme levels.

Using atomic absorptiometry, significant ammounts of cobalt were found in the secondary focus and other cortical areas, and it is suggested that the secondary focus may not be the biochemically "pure" model it has previously been assumed to be. The diffusion or other transport of cobalt from the implant area is discussed in terms of the possible effects of cobalt poisoning of the enzymes assayed.

P.T.O. P

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SUMMARY

I The neurological basis of the epileptic condition in one experimental model, the cobalt-implanted rat has been investigated with a view to attempting to link the epileptic condition to one or more of the sub-groups of central neurones, as defined in terms of the neurotransmitter. It was hoped that any evidence of preferential involvement during the development of epilepsy might serve as a basis for farther analysis and/or clinical work.

II Many authors have suggested that the seizure threshold in many models of epilepsy may be related to alterations in the activity of central monoaminergic systems. Therefore a group of related enzymes, neurotransmitters and their metabolites were assayed in different areas of the brain including the primary and secondary foci at a range of times after the implantation of a cobalt pellet into rat frontal cortex, a procedure that initiates an epileptic process. The time course of any changes found was compared to the development of the epileptic state as measured behaviourally and by computer analysis of the EEG. These studies were a continuation of the large body of work already carried out in the M.R.C. Brain Metabolism Unit concerned with monitoring biochemical changes in the cobalt-implanted rat.

III The basic programme of work for this thesis began very broadly, employing a wide spectrum of tests as a method of screening as large a range of parameters as possible; following the first detection of abnormalities a second series of tests was employed to examine more closely the anomalous data, particularly that related to the serotonergic system. Because of this method of approach, and also because each tissue sample repres-

ented one rat which had been operated on, and subsequently recorded and observed for periods of up to 100 days, the number of rats sampled in certain tests, as recorded in the results section, was necessarily limited. This problem was exacerbated by the operated rats' increased susceptibility to respiratory disease and respiratory failure during the first month after operation.

IV The mechanisms involved in the development of the secondary focus were investigated. Using flameless atomic absorptiometry, it was shown that cobalt was present in the cortex contralateral to the implant at 6 days post-implant. Treatment of the animals during the first week post-implant with acetoxycycloheximide was shown to depress rates of cerebral protein synthesis by 60%, but did not affect the development of the secondary focus, as measured in the EEG. These findings suggested that the formation of the secondary focus may be influenced by cobalt in the contralateral cortex, and seem to argue against the theory of a "learning" process contributing to the developing electrical changes in the secondary focus.

V Gamma aminobutyric acid transferase (GABA-T), an enzyme associated with gabaminergic neurones, was found to be initially reduced in both primary and secondary epileptic foci, suggesting that gabaminergic neurones may be in some way involved in the development of the epilepsy.

VI Various parameters of the central catecholaminergic nervous systems were examined. Levels of enzyme activity of tyrosine hydroxylase (TOH), catecholamine-O-methyltransferase (COMT), monoamine oxidase (MAO) were also reduced at an early stage in both primary and secondary focal areas, and with the exception of a temporary

increase in MAO and COMT mainly in the area of the primary focus thought to be due to a glial reaction, were still subnormal in both foci at 30 days post-implant but had returned to normal by 100 days. Changes in cortical protein content were also noted, the protein/wet weight ratio becoming reduced at 3 days after cobalt implantation in the primary focus and at 6 days in the secondary, and remaining low for at least 30 days. The rapidity of the changes suggested that the changing protein/wet weight ratio was an indication of the development of cerebral oedema, and all of the above enzyme data were therefore expressed in relation to total protein rather than per wet weight of tissue.

VII TOH activity in mid-brain sections containing the locus coeruleus, the location of the cell bodies of the noradrenergic neurones, was unaffected in epileptic rats, as were cortical concentrations of noradrenaline (NAd). Striatal and cortical levels of 4-Hydroxy-3-methoxyphenylglycol (HMPG), a cerebral metabolite of NAd, showed increases on the lesion and contralateral sides by 8 days post-implant, but had returned to normal by days 15-20 post-implant. A cerebral metabolite of DA, homovanillic acid (HVA) in the striatum was also raised at 4 days, and had still not normalised at 20 days. To account for the apparently paradoxical situation of low enzyme activities (TOH, MAO and COMT) occurring together with raised metabolite concentrations, a reduction in transport of the metabolites from the brain is postulated.

VIII The serotonergic (5-hydroxytryptoaminergic) system was also examined. Various parameters remained unaffected, including cortical 5-HT (5-hydroxytryptamine) concentrations, and rates of cortical 5-HT uptake. In the cortex, levels of tryptophan, the amino acid precursor

of 5-HT, were low at 6 days post-implant and elevated to above normal by 30 days. Concentrations of the 5-HT metabolite, 5-hydroxyindole acetic acid (5-HIAA) fell at 15 days post-implant around the primary focus and at 20 days in the secondary focus, and had returned to normal at 100 days. There was evidence of a transient increase in cortical 5-HIAA at 4 days post-implant, and a more consistent elevation of striatal 5-HIAA levels, lasting until 24 days. These findings, like those of increased catecholamine metabolite concentrations which occurred over a comparable period, are also suggestive of a block of metabolite transport from the brain.

IX Tryptophan hydroxylase activity in sections of mid-brain containing the raphe nuclei which contain the cell bodies of the serotonergic neurones was reduced at 20-25 days. In an attempt to analyse further the reduction in cortical 5-HIAA and mid-brain tryptophan hydroxylase activity, a group of epileptic rats was given loading doses of tryptophan. The subsequent increases in cortical and striatal 5-HT and 5-HIAA did not differ significantly from control animals.

X A survey of the literature on the relationship between serotonergic tone and the seizure threshold is presented, and on the basis of this data and the results detailed in this thesis the hypothesis is put forward that the serotonergic system responds in a protective manner to the epileptic condition by reducing turnover of 5-HT; it is also suggested that the degree of serotonergic activity may be critical in determining the seizure theshold. This hypothesis has been tested by examining the effects of drugs, known to act on the serotonergic system, on the EEG of the cobalt-implanted rats. Preliminary observations have indicated that increasing cortical 5-HT concentrations by tryptophan loading aggravates epileptic behaviour, but when

clorgyline, an MAO inhibitor, is given in conjunction with the tryptophan loading, all epileptic signs were reduced. However the development of epilepsy in cobalt-implanted rats was found to be unaffected by a long-lasting central 5-HT depleting agent, 5,7-dihydroxytryptamine, and furthermore tolazoline, a drug thought to include a blocking effect on central 5-HT receptors amongst its actions was equally ineffective as an antiepileptic. The theory of the relationship between serotonergic tone and the seizure threshold is re-evaluated in the light of these observations.

MATERIALS

1. All inorganic salts used were of "analytical reagent" grade or better.
2. Water used was all distilled in all-glass apparatus and deionised using Elgastat equipment.
3. The sources of the chemicals used in the experimental work are listed below.

Acetic anhydride	Sigma (redistilled)
acetone	B.D.H.(")
acrylic resin	Simplex, cold-curing
S-Adenosyl-L-[methyl-C^{14}] methionine	Amersham
4-Amino-n-(U-C^{14}) butyric acid	"
aluminium oxide, Woelm neutral	Woelm Eschwege, Germany
Ampholines	LKB Produktor, Uppsala
bone wax	Ethicon
Bovine serum albumen	Sigma fracn. IV
n-butanol	B.D.H. (spectroscopy grade)
Catalase	Boehringer Mannheim
cobaltous chloride	Sigma
cobalt powder (Mesh 200)	"
Coomassie Blue G250	Gurrs
1:2 dichloroethane	B.D.H. (redistilled)
Dichloromethane	B.D.H. "
3,4-dihydroxyphenylglycol sulphate	Sigma
Dithiothreitol (Clelland's reagent)	Sigma
Dopamine creatinine sulphate	Sigma
Ethanol	B.D.H. (redistilled)
Ethanolamine	B.D.H. (spectroscopy grade)

Ethyl acetate	Reeve Angel Scientific (D.T. grade)
Ethyl butyl ketone	Koch-Light
Formaldehyde (40%)	B.D.H.
Gelatine	Gurrs
Halothane (Fluothane)	I.C.I.
Helicase	Industrie Biologique Francais
n-heptane	B.D.H. (spectroscopy grade)
Hexachlorocyclohexane	Koch-Light
Homovanillic acid	Sigma
Hydrochloric acid (concentrated)	B.D.H. 'Aristar'
3-hydroxybenzyloxyamine (N.S.D.-1055)	Smith and Nephew
4-hydroxy-3-methoxyphenylethylene glycol	Sigma

Krebs-Ringer bicarbonate buffer (g/l): NaCl, 6.92; KCl, 0.35; $MgSO_4.7H_2O$, 0.29; $CaCl_2.6H_2O$, 0.28; KH_2PO_4, 0.162; $NaHCO_3$, 2.10; glucose, 1.8.

2-mercaptoethanol	Koch-Light
Michel clips	Aesculap 12 x 3 mm.
Noradrenaline creatinine sulphate	Sigma
O-phthaldialdehyde	Schwarz-Mann
Polybactrin	Calmic Ltd.
Serotonin creatinine sulphate	Sigma (Analar grade)
Sodium tetraphenyl borate	Merck
Tetrahydrobiopterin	Roche (a gift)
Toluene	Koch-Light
Trifluoroacetic anhydride	Sigma (redistilled)
Tris (hydroxymethyl) methylamine	B.D.H.
Triton X-100	Rohme and Haas

Triton X-100 scintillant:

1ℓ toluene, 0.5ℓ triton X-100	
4.0 g PPO (2,5, diphenyloxazole)	N.E.N.
0.1 g POPOP (1,4 di (2-(4-methyl, 5 phenoxazolyl)) benzene)	N.E.N.
L-tryptophan	Sigma (E' grade)
L-[5-^{3}H]-tryptophan	Amersham
Tyramine-1'-C^{14} hydrochloride	"
L-(side chain -2,3-H^{3})tyrosine	"

Appendix I. Material used in recording unrestrained, conscious rats:

Connectors;	Amphenol (spring type)
braided leads:	Grass

METHODS

This section contains details of all the methods used in this thesis. It includes the preparation of the experimental animals, the subsequent recording of ECoG's, dissection techniques and the methodology of the biochemical assays.

A. Mechanical procedures

I. Method of cobalt implantation.

II. Recording techniques.

III. Collection and storage of brain tissue samples.

IV. Dissection techniques.

V. General preparation of brain tissue samples for enzyme assay.

B. Enzyme assays

I. Y-aminobutyric acid (GABA) transaminase (GABAT).

II. Tryptophan hydroxylase (Tryp. OH).

III. Tyrosine hydroxylase (TOH).

IV. Catechol-O-methyltransferase (COMT).

V. Monoamine oxidase (MAO).

C. Assays of neurotransmitters, and their precursors and metabolites.

I. Noradrenaline and dopamine (NAd and DA).

II. Homovanillic acid (HVA).

III. 4-hydroxy-3-methoxyphenylglycol (HMPG).

IV. Tryptophan.

V. 5-hydroxytryptamine and 5-hydroxyindoleacetic acid (5-HT and 5-HIAA).

D. Other methods

Ia. Protein assay.

Ib. Estimation of rates of cerebral protein synthesis.

II. Rate of 5-HT uptake into brain.

III. Cobalt assay.

IV. Tryptophan loading.

A. MECHANICAL PROCEDURES

The model used in this series of experiments to study the biochemistry of an epileptic type of brain malfunction consisted of rats which were given a cobalt implant into the cortex. This implant induced the formation of an epileptic focus first on the ipsilateral side and, in due course, on the contralateral side. Subsequent to the cobalt implantation the development and persistence of these epileptic foci were monitored by EEG recordings. Changes in the concentrations of putative central neurotransmitters and of their synthetic and catabolic enzymes were also monitored, both in the foci and in other brain areas, with a view to possibly relating the electrophysiological and biochemical events in some unifying hypothesis.

I. Method of cobalt implantation into rats' brains with subsequent recording of electrocorticogram (ECoG).

The cobalt was implanted as a suspension in solidified gelatine, prepared according to the method of Fischer et al. (373). Cobalt powder (5ml, Sigma, mesh 200) was added to 5ml 5% (w/v) aqueous gelatine solution at 56°C. The suspension was thoroughly mixed, and allowed to settle for 2 min. Two ml of the supernatant were decanted to remove fines and the remaining 8ml suspension poured over horizontal

microscope slides to form a layer approximately 0.75 mm thick. After cooling, the cobalt gelatine films on the slides were immersed in acetone for 2h to dehydrate the gelatine, and then placed in a desiccator containing paraformaldehyde for 2h to allow fixation of the gelatine. The slides were then steeped in distilled water and finally stored in 80% (v/v) ethanol.

Slides were removed from the alcohol 5-10min. before use, to allow the alcohol to evaporate from the gelatine film. Discs (1mm diameter) for implantation in rat cortices were cut as required from the cobalt-gelatine film with a 1mm punch. The cobalt content of such a disc was estimated from the dilution of the cobalt powder used to be of the order of 500-750µg. "Glass implants" were prepared using the identical procedure, using ground glass (Mesh 175, Sigma) in place of the metallic cobalt.

Operative technique

The following procedures concerning the operation and subsequent recording of the EEG from the cobalt-implanted rat are all derived from work carried out in the MRC Brain Metabolism Unit at Edinburgh University (References 125, 354 and 567).

All surgical procedures were carried out under aseptic conditions. Male Piebald Virol Glaxo (PVG) rats ($2\frac{1}{2}$ months old, 200-250g) were anaesthetised with halothane (Fluothane, ICI) in oxygen (2.5 l/min). A halothane concentration of 11% was used for induction and 7-9% for maintenance of anaesthesia. The scalp was shaved using an electric hair trimmer, and swabbed with 5% iodine solution in ethanol. A mid-line incision (1.5-2.5cm long) was made on the scalp, and the underlying fascia was reflected to expose the outer surface of the

skull. After locating the coronal and sagittal sutures, the skull was trephined, the holes being centred at a point 2 mm from each suture in the right anterior quadrant. The hole was made with a dental drill (Renda model RA/21) and a round No. 6 burr (Ash), care being taken not to damage the underlying dura. Haemorrhage from the skull did not constitute a major problem. A cobalt implant was made into the frontal cortex as follows. At the site of implantation the dura was split with the tip of a sterile 23-gauge needle. A disc of cobalt-gelatine was cut from a prepared film using a 1mm diameter tissue punch fitted with a plunger, and inserted into the superficial layers of the underlying cortex. The punch was so tapered that it protruded a set distance through the standard hole drilled in the skull with a No. 6 round burr, thus controlling to some extent the depth of the implant. The hole in the skull was then sealed with bone wax and the whole operative area was sprayed with antibiotic (Polybactrin). The incision was closed with Michel clips. The whole operative procedure took between 10 and 15 min.: no special post-operative care was found to be necessary. The Michel clips were removed 7 days later when the wound had completely healed.

In the case of an animal whose EEG was to be recorded, the operation procedure was modified to include the fitting of permanent electrodes.

1) Using the same equipment and pre-operative procedures as above, a curved cranial incision (2.3 cm long) was made through the scalp close to the left eye. The skull was cleared, and the coronal, sagittal and naso-frontal sutures located and used as landmarks to ensure consistent placement of the recording electrodes. The skull was trephined, pairs of holes being centred 2 mm on each side of the sagittal and coronal sutures. Cobalt-gelatine implantation into a

frontal cortex was carried out as above, and then the burr-holes were tapped with an 8 BA tap (or alternatively counter-sunk with a no. 9 round burr). Specially constructed stainless steel screws were inserted into the holes, to act as extradural recording electrodes (374). It was found necessary to secure the screws to the bore using cold-curing acrylic resin (Simplex), and to aid this purpose a groove had been cut round the body of the screw. One of the electrodes was placed directly over the implant, and one in each of the other three holes. Fig. 1 shows the design of the screws and their placement in the skull. The operation area was then sprayed with Polybactrin and the skin flap replaced after four holes had been punched in it so that it fitted over the electrodes and allowed them to protrude externally. The wound was then closed with Michel clips. This whole procedure took between 30 and 45 min; again, no special post-operative care was found to be necessary. In general screws have remained firm in situ for periods of 6 months or more.

II. Recording Techniques

EEG recordings were made from the unrestrained, conscious rat. Spring-connectors (Amphenol) attached to braided wire (Grass) were fitted into the hollow electrodes, and brain potentials were recorded on a Grass Model 7 Polygraph, the controls of which were set as recommended for the recording of the human EEG. The EEGs were simultaneously recorded on tape using a Tandberg 1.R. series 100 tape recorder, with tape speed of $1\frac{7}{8}$ inches per s. and input range of 2v. The tapes were subsequently used for computer analysis and 'spike' counting (567).

Recording sessions were standardised as far as possible, particularly with regard to the time of day and the sequence of recording the individual animals in any group, as there had been reports (52, 576) of diurnal fluctuations in the seizure threshold of various epilepsy models. To allow time for the dissipation of the stress induced in the animals by handling and their transfer to the novel environment of the recording cage, an interim period of 10 min. was allowed between electrode connection and subsequent recording. The recording was carried out over the subsequent 10 min. period, the ECoG of the lesioned and contralateral cortices being recorded simultaneously. For the purpose of the experiments reported in this thesis, the EEG recordings were analysed and described in terms of the number of spikes produced in a given time.

The tape recordings were analysed using a computer programme developed by Hill and Townsend (567), which operates by dividing EEG phenomena into spikes or waves, on the basis of the peak angles of all the wave forms. This programme has been designed to monitor 10 min. recordings, and gives a figure for the total spike count over the 10 min. The values cited in the relevant sections of the thesis are given as spikes/min., averaged from a 10 min. recording.

III. Collection and storage of brain samples for biochemical analyses

Groups of experimental animals were stunned and decapitated at set times, from 5 to 100 days, after cobalt implantation. The brains were removed immediately, wrapped in aluminium foil, and stored in liquid nitrogen until all the rats in any one experiment had been sampled. In practice no tissue sample was left in liquid nitrogen for longer than 25 days before being used for analysis. In those series containing

"long-term" (85-100 days post-implantation) animals, these latter animals were operated on 2-3 months before the 'short-term' (30 days) animals in the particular experiment. Generally, one control rat was killed with each experimental group, ensuring that the control brain samples were stored over similar lengths of time as experimental brain samples. 'Control animals' here refers to both unoperated littermates of the experimental animals, and to sham-operated animals. These latter were operated on exactly as the experimental animals, but no cobalt was placed into the cortex. Some of this latter group were given 'glass-in-gelatine' implants (prepared according to Fischer (373). The type of control used in each experiment is stated in the report of the results.

IV. Dissection of brain samples for the biochemical analyses

A) Cortex and Caudate

When the collection of brains from a series of experimental animals had been completed, the brains were removed one at a time from the liquid nitrogen and placed on a 2mm thick glass plate resting on a mixture of solid CO_2 and crushed ice. Tissue samples were cut as the tissue thawed and became workable. In the case of samples of tissue containing the site of implant, all visible pieces of cobalt-gelatine were removed as was any calcified tissue.

Cortical samples were dissected as follows. The brain was placed on its ventral surface on a glass plate resting on a mixture of crushed ice and solid CO_2. The cerebellum was removed, and the forebrain bisected. The curved tip of a micro-spatula was then inserted into the lateral ventricle, passed rostrally and caudally and the cerebral cortex reflected laterally. For some of the less sensitive assays (generally those depending on fluorimetric methods)

the cortical tissue thus obtained was bisected coronally to give samples weighing 40-60 mg. For the more sensitive radiometric assays, smaller samples (15-25mg) were obtained using a 4 or 5 mm diameter stainless steel punch to cut discs of tissue centred on the sites of the primary and secondary lesions in the cortical slabs.

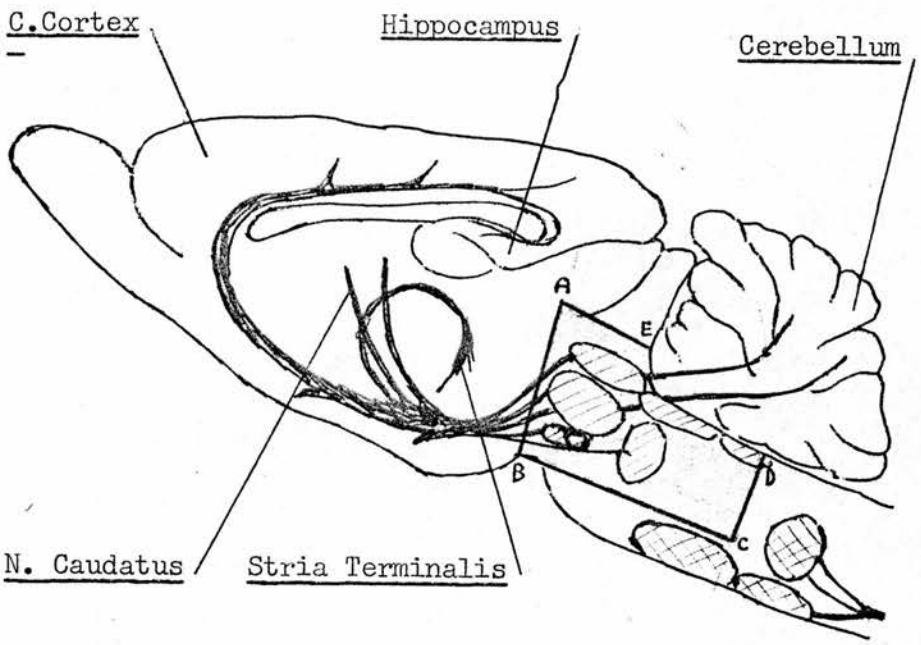
Samples of the caudate nuclei were prepared as follows. After placing the brain on its ventral surface, the forebrain was cut through coronally 8 and 10 mm from the occipital cortical extremity. The 2 mm thick forebrain section thus obtained was then laid on the glass surface, and discs of approximately 5mg from the caudate nuclei were cut out using a 2 mm diameter stainless steel punch

B) Raphe nuclei

The method developed was based on a diagram (Fig. 2) of the central 5-HT systems published by Fuxe et al (552). At predetermined times after cobalt implantation, a rat was stunned and decapitated. The brain was rapidly removed and placed on its ventral surface on an aluminium block cooled to 0°C on a bed of crushed ice. The cerebellum was then gently separated from the cortices, peeled back along the midbrain and removed. The spinal cord was cut at the level of the median cerebellar penduncles. A scalpel blade was inserted between the occipital cortices and the pre-optic tecta, and with this cut the forebrain removed. The optic and pre-optic tecta were then severed with a horizontal cut at the level of the third ventricle. Finally a cut was made along the ventral surface of the midbrain from the level of the pyramids caudally, removing the ventral third of the midbrain section. The section remaining contained the rostrally

Fig. 2

Serotonergic systems within the rat brain



ified from Fuxe, Hokfelt, Olson, and Ungerstedt '74.

Anteriorly projecting cell groups
Posteriorly projecting cell groups

Area disected.

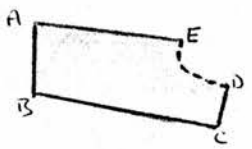


TABLE 1 Tryptophan hydroxylase activity (n.mol. 5-hydroxyindoles/g brain/h) and 5-HT concentrations (ug 5HT/g brain) in sections of mid-brain.

Using the dissection techiques described in Methods section A IV, a range of mid-brain sections were cut as follows:

A Sections of whole mid-brain - that segment remaining after removal of the cerebellum, section of the spinal cord and removal of the fore-brain.

B As in A, but after removal of the optic and pre-optic tecta.

C The severed optic and pre-optic tecta.

D As in B, after removal of the ventral third of the mid-brain. This section was used in all succeeding experiments.

Enzyme activity (n,mol. 5-hydroxyindoles produced/g brain/h)

A	B	C	D
1.2	2.3	nil	2.3
1.4	2.7	nil	2.8
1.6	2.1	nil	2.3
1.6	2.1	nil	2.6
1.45 ± 0.16	2.0	nil	2.2
	2.24 ± 0.24		2.44 ± 0.22

Amine concentrations (µg 5HT/g brain)

A	B	C	D
1.11	1.31	0.29	1.15
0.93	1.53	0.37	1.08
0.87	1.27	0.22	1.21
0.95	1.3	0.28	0.94
0.97 ± 0.1	1.64	0.29 ± 0.06	0.92
	1.41 ± 0.17		1.06 ± 0.13

projecting raphe nuclei, and left out the caudally-projecting nuclei. As all cortical serotonergic innervation derives from the anterior nuclei, it was thought that any responses in these nuclei to cortical events would be diluted if all nuclei were included in the sample for analysis. To check on the accuracy of this dissection, tryptophan hydroxylase activity (Methods BII) was assayed in various parts of the midbrain. It was established that the method described above did in fact provide a section of tissue with the highest enzyme activity/weight ratio (Table 1), suggesting that the anterior raphe nuclei had been effectively sampled.

The typical weight of this section was 165-185 mg. Dissected samples were stored in liquid nitrogen until all had been collected from a series of animals killed at different times after implantation. Tryptophan hydroxylase assays were then all carried out on the same day, necessitating a period of storage, in some cases, of up to 25 days. As one control animal was killed with every group of experimental animals and the control group showed in itself little "between animal" variation, it was assumed that no significant loss of enzyme activity occurred over this period.

V. Preparation of brain extracts for the estimation of the activity of various enzymes

The activity of the following enzymes, tyrosine hydroxylase (TOH), monoamine oxidase (MAO), catechol-O-methyl transferase (COMT) and gamma-aminobutyric acid aminotransferase (GABAT), were estimated in samples of rat brain using radiometric assays. Samples were prepared for assay as follows.

All procedures were carried out at 0-4°C. Weighed samples of brain, weighing typically between 20 and 30 mg (either freshly removed or from storage for up to 25 days in liquid nitrogen) were homogenised in 10 volumes of buffer in an all-glass homogeniser for 2.5 min. at 3,000 r.p.m. The homogenising buffer consisted of 0.1M K_2HPO_4/NaH_2PO_4 buffer pH 6.8 containing 1 mg 2-mercaptoethanol/ml, and made 0.5% v/v with Triton X-100. Homogenates were transferred to 1.5 ml reaction tubes and centrifuged at 12,000 g for 10 min. at 0°C in an Eppendorf Zentrifuge. Samples of the clear supernatant were used for the radiometric assays of the enzymes mentioned above, and for protein estimation. This tissue preparation procedure was a modification of that of Hendry and Iverson (353) in their assay of tyrosine hydroxylase in rat brain.

B. ENZYME ASSAYS

I. Estimation of γ-aminobutyric acid (GABA)-transaminase activity of brain tissue

This assay was based on the method of Hall and Kravitz (574). The method basically consists of the incubation of a preparation of brain with ^{14}C-labelled γ-aminobutyric acid (GABA under suitable conditions, in which the amino group of the GABA is catalytically transferred to an acceptor, α-ketoglutaric acid, by the transaminase enzyme. For quantification, the unused labelled substrate is removed by adsorption on a cation exchange resin, and the unabsorbed deaminated reaction products from the GABA, succinate and succinic semialdehyde, estimated by scintillation counting. Excess succinate is added to the incubation mixture to reduce further metabolism of the labelled succinate formed in the main reaction.

5µl of the supernatant fraction of samples of brain prepared as described in Methods section A, part V, were added to an incubation mixture, total volume 50µl containing the following:

150 nmol unlabelled GABA, 100 nmol α-ketogluarate, 2 µmol 2-mercaptoethanol, 100 nmol NAD, 100 nmol sodium succinate, 20 nmol pyridoxal phosphate, 5.0 nmol ^{14}C-GABA (4-amino-n-(U-^{14}C) butyric acid, sp. act. 150 mCi/mmol, and 5 µmol potassium glycylglycine, which last was previously adjusted with 5M KOH to pH 8.5 (glass electrode). The final pH of the incubation mixture was close to 8.3 (glass electrode).

Incubations were carried out in stoppered 1.5 ml polycarbonate tubes at 37°C for 1h. The reaction was stopped by adding 5 µl trichloroacetic acid (0.4g/ml) and the whole reaction mixture was then applied with washing with 50µl distilled water to a cation exchange column, containing Dowex-50x2, 100-200 mesh. The columns, 3cm x 0.5cm dia., were washed with 20 ml 25 mM HCl before use. After application of the incubation mixture the resin column was washed with 5 ml distilled water, and the effluent containing the reaction products of the incubation, was collected. After mixing, 1 ml was added to 10 ml Triton scintillant for subsequent counting in a Hewlett-Packard scintillation counter for 10 min., external ratio mode. A second 5 ml wash of distilled water of such solumns was found to contain very little radioactivity (100-150 cpm/ml).

The Dowex column quantitatively retained GABA, as measurdd by the very low values of tissue blanks (200-500 cpm), obtained from incubates prepared by adding the trichloroacetic acid to the tissue extract before the mixture containing the substrate. All tissue

Fig. 3

GABA transaminase activity in cortical homogenates as a function of pH of incubation

Enzyme activity expressed as percentage of mean activity at pH 7 (330 ± 13.3 (6) n.mol. GABA transaminated/g protein/h.). Vertical bars show standard deviation of 6 replicate samples at each pH value.

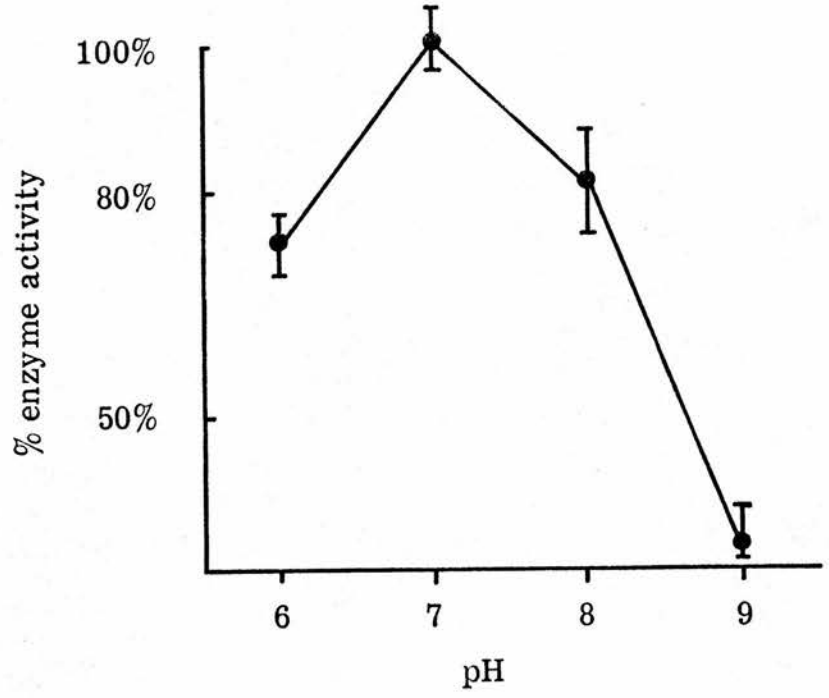
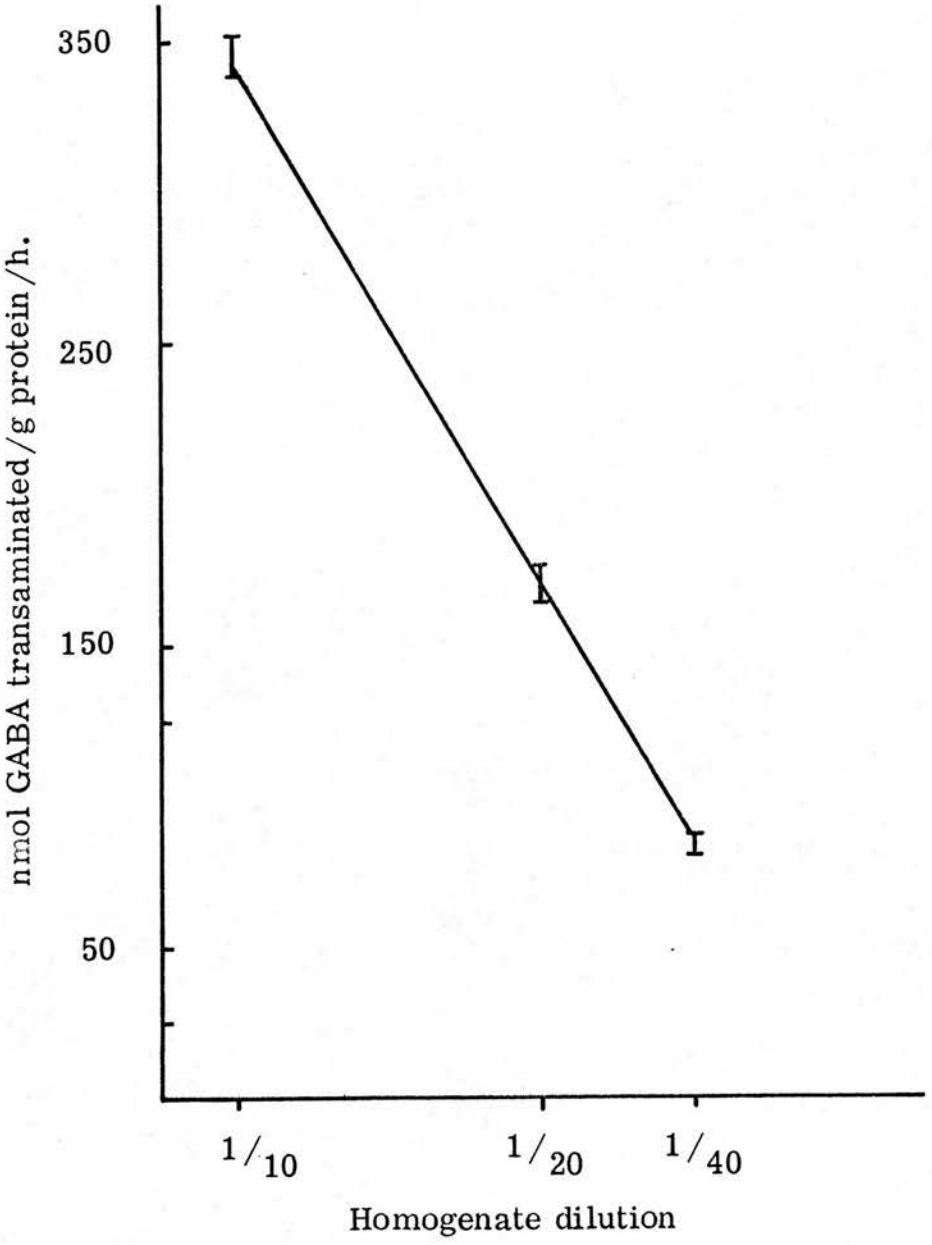


Fig. 4

Linearity of GABA-transaminase activity in relation to enzyme concentration in cortical homogenates



1. A fixed volume of each homogenate was added to a standard incubation mix.
2. 1/10, 1/20 etc. refers to 1mg. tissue in 10, 20, etc. µls homogenising buffer.
3. All points on graph refer to $\bar{x} \pm$ SD' for groups of 8 replicate samples.

samples giving counts less than twice those of the "tissue blanks" were assigned 'nil' values.

The specific activity of the ^{14}C-GABA in the reaction mixture was 4.84 mCi mmol, close to the specific activity of the ^{14}C-GABA originally used by Hall and Kravitz (574). As the specific activity of the ^{14}C-GABA from Amersham was so much higher than the ^{14}C-GABA they used, a dilution with unlabelled GABA was decided on to make the assay more economical. The addition of 150 nmol unlabelled GABA brought the overall concentration of GABA in the reaction mixture to 2.82mM, which Hall and Kravetz suggested ensured enzyme saturation conditions.

Since both succinic semi-aldehyde and succinate are present in the column effluent, this method is therefore not affected by changes in the activity of succinic semialdehyde dehydrogenase in the tissues being analysed. Further degradation of the formed succinate with concomitant loss of ^{14}C was reduced to a minimum by the presence of excess added succinate in the incubation mixture. The validity of the enzyme assay as described was checked by establishing a linear relationship between enzyme activity and the concentration of the tissue homogenate (Fig. 4) and by establishing that the pH profile of the enzyme matched already existing data (Fig. 5).

II. Estimation of tryptophan hydroxylase (EC 1.14.36) activity in rat brain

The method of estimation was based on that of Baumgarten *et al* (243) which was itself a modification of the procedure reported by Friedman *et al* (244). Basically, a homogenate of brain containing the enzyme tryptophan hydroxylase is incubated with L-tryptophan, and after the reaction has been terminated 5-HT and 5-HIAA are

estimated fluorimetrically in the incubation mixture using the method of Bogdanski et al (240).

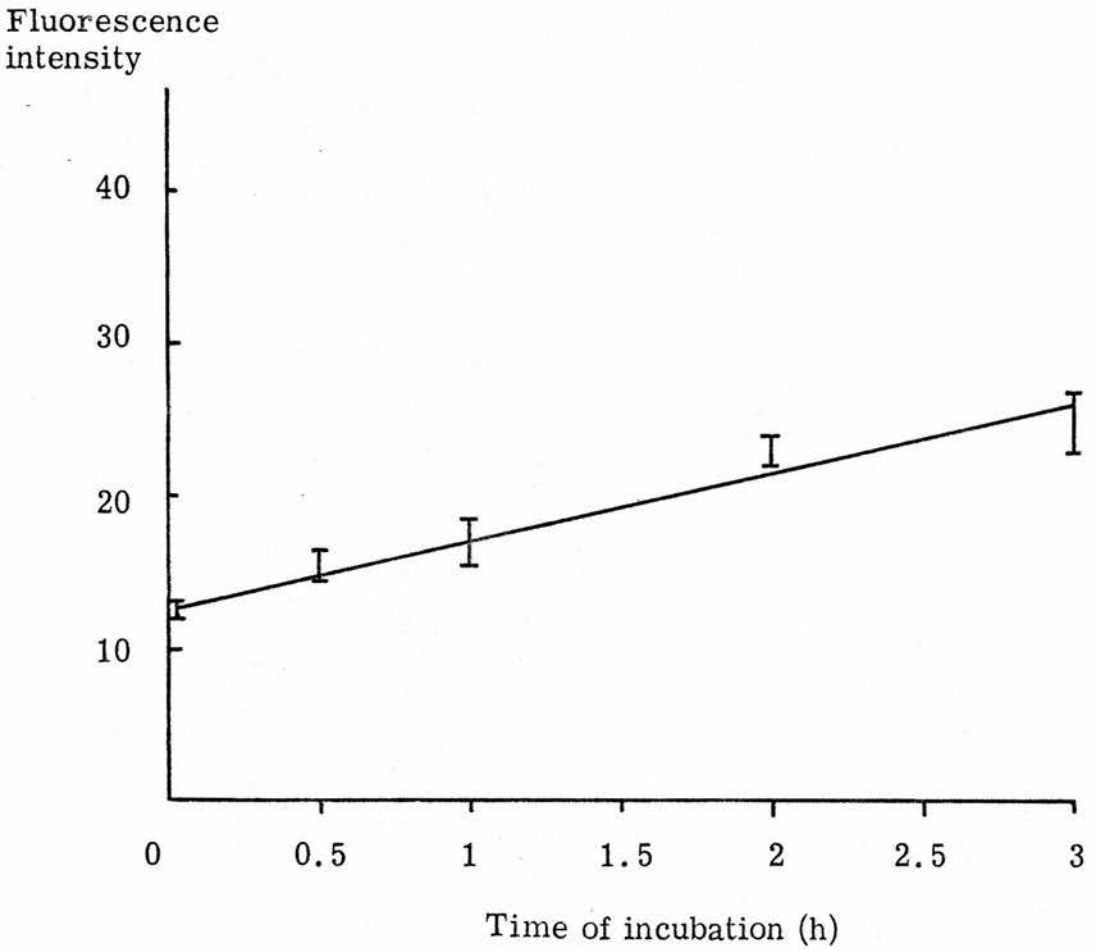
The estimation procedure detailed here proved sufficiently sensitive to assay tryptophan hydroxylase activity in the raphe nuclei, but not in the small cortical samples (15-25 mg) that were being used in the assays of other enzymes by radiometric techniques.

Thawed samples containing the anterior raphe nuclei dissected as described in the methods section A, part IV, were weighed (180.5 ± 9.2 mg, $\bar{x}$ ± SD for one series of 10 samples) and homogenised immediately in all-glass equipment at 2,000 r.p.m. for 2 min. in 5 vol. (5 µl/mg tissue) of 33 mM sodium phosphate buffer pH 7.4 containing 2 mmol dithiothreitol per litre. Portions of the homogenates were transferred to 1 ml polycarbonate reaction tubes and centrifuged for 5 min. at 12,000 g using an Eppendorf 'Zentrifuge'. All steps were carried out at 0-4°C. Duplicate samples, 100 µl, of the supernatant, were taken for subsequent estimation of enzyme activity. Each supernatant sample was transferred to a 1 ml reaction tube, containing a mixture of 1 µl catalase solution (55,000 units activity/ml), 10 µl 2 mM L-tryptophan and 5 µl 10 mM tetrahydrobiopterin. The L-tryptophan and tetrahydrobiopterin were dissolved in distilled water.

The reaction mixture was incubated at 37°C for 2 hr after which time the reaction was stopped by adding 10 µl 6 M-perchloric acid. The precipitated protein was removed by centrifugation for 2.5 min. at 12,000 g. Portions, 100 µl, of the supernatant were removed and stored at -20°C until required for assay of the formad 5-hydroxyindoles. Immediately prior to fluoroassay, 25 µl conc. HCl (B.D.H. Aristar)

containing freshly dissolved ascorbic acid (0.5 mg/ml) was mixed with each thawed sample. The procedure of the fluoroassay was standardised as far as possible. All samples were kept on ice until immediately prior to reading, when the addition of the HCl and subsequent transfer of the solution to the micro-cuvette, and the fluorimetry were timed to the second, using stop-clocks, as follows: mixing with HCl, 2 sec., transferral to the micro-cuvette at 4 sec., and activation scans of samples began at 7 sec. The conditions of fluoroassay were identical to those described in the assay of 5-HT and 5-HIAA.(Methods C,V). The amount of 5-hydroxyindoles formed was calculated from a standard curve of the fluorescence of authentic 5-HT added to complete but unincubated reaction mixtures. Two pairs of duplicate samples were taken from each homogenate of mid-brain. One pair was incubated as above for 2h. For estimation of the endogenous 5-hydroxyindole levels the other pair was stopped at incubation time zero, which in practice gave readings of slightly under half of those of the experimental samples. The amount of 5-hydroxyindoles in each pair was then calculated, and the endogenous 5-hydroxyindole values were subtracted from the post-incubation values to give a final figure representing 5-hydroxyindoles formed during the 2 h. incubation. Although in the original method of Friedman *et al* (244) it was suggested that a decarboxylase inhibitor should be used to prevent further metabolism of the formed 5-hydroxytryptophan (5-HTP), Baumgarten *et al* (243) found this unnecessary. In their hands the fluoroassay of total 5-hydroxyindoles (5-HT, 5-HIAA and 5-HTP if any) proved an accurate and relaible index of tryptophan hydroxylase activity. They also suggested that by not using an MAO inhibitor, the reaction's equilibrium would be shifted towards an

Linearity of tryptophan hydroxylase assay with time



1. The ordinate scale refers to units of fluorescence as measured using the Perkin-Elmer spectrofluorimeter MPF-2A, sensitivity scale 6, and corrected for background fluorescence (Methods CV).
2. The point on the curve at time zero represents fluorescence attributable to endogenous 5-hydroxyindoles in the raphe nuclei.
3. All values are given as $\bar{x} \pm SD'$, and are all derived from groups of 10 duplicates, except for the group at time zero, which only consisted of 7 samples.

Fig. 6

Tryptophan hydroxylase activity in mid-brain homogenates as a function of pH of incubation

Enzyme activity expressed as percentage of mean activity at pH 7.5 (2.9 ± 0.2 (6) n.mol 5-hydroxyindoles (5-HT equivalents) produced/g protein/h.). Vertical bars show standard deviation of 6 replicate samples at each pH value.

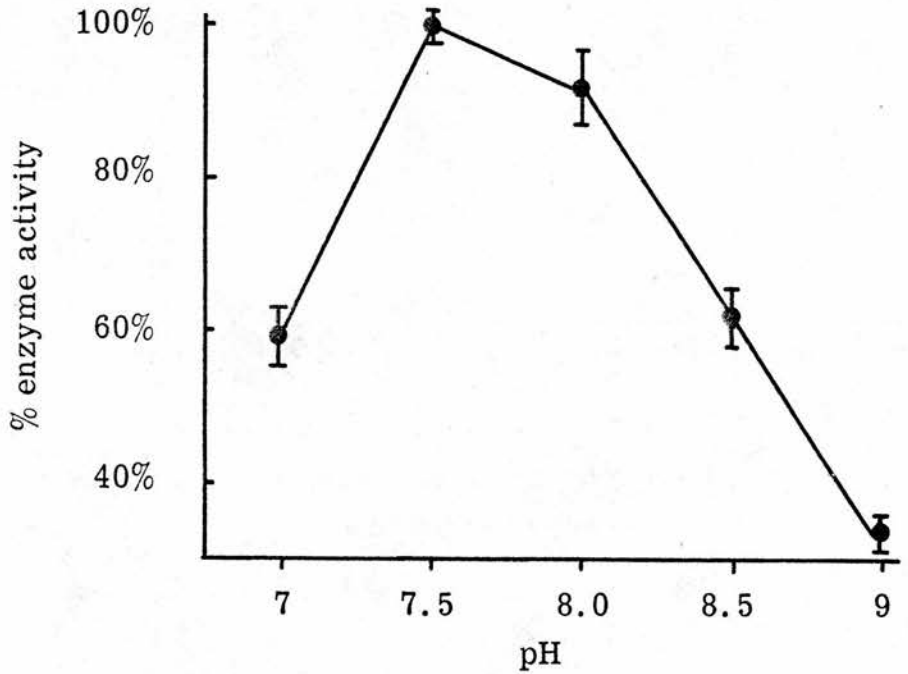
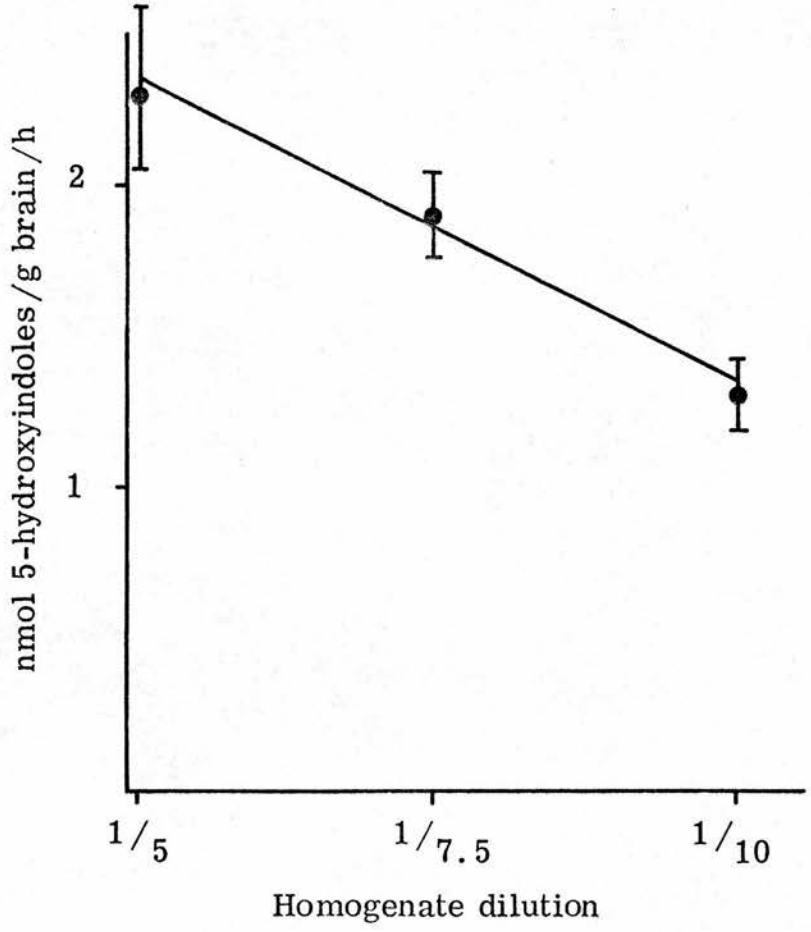


Fig. 7

Linearity of tryptophan hydroxylase activity in relation to enzyme concentration in cortical homogenates



1. A fixed volume of each homogenate was added to a standard incubation mix.
2. 1/5, 1/7.5 etc. refers to 1mg tissue in 5, 7.5 etc. µls homogenising buffer.
3. All points on graph refer to $\bar{x} \pm$ SD' for groups of 8 replicate samples

increase in the overall rate of hydroxylation, in accordance with the principle of Le Chatelier. However, with hindsight I now regret not having used an MAO inhibitor in view of the different relative molar fluorescence of the 5-hydroxyindoles (238), and the changes in MAO activity that develop in the cobalt-implanted rat.

The final substrate concentration of L-tryptophan (200 μM) was a compromise. At this concentration of L-tryptophan the sensitivity of the fluoroassay of 5-HT was diminished 3-fold, by a combination of an elevated background fluorescence plus a degree of fluorescence quenching. But substrate concentrations also had to be high enough initially to provide saturating conditions throughout the 2 h. incubation. Using previously published data on rates of tryptophan hydroxylation in mid-brain (18), calculation indicated that in the 2 h. incubation period insufficient of the added substrate would be metabolised to produce a sub-saturating condition. This was supported by finding that the hydroxylation did appear to develop linearly over the 2 h. of the incubation (Fig. 5). The fluorescence readings at time zero in this table refer to endogenous mid-brain 5-hydroxyindole concentrations, approximately 1.5 μg/g wet weight tissue.

The validity of the enzyme assay as described above was also checked by establishing a linear relationship between enzyme activity and the concentration of the tissue homogenate (Fig. 6) and by establishing that the pH profile of the enzyme as assayed here matched existing data (Fig. 7).

II. Unsuccessful assay of tryptophan hydroxylase using a radiometric method

As indicated in the previous section the method for estimation of tryptophan hydroxylase activity, involving the fluorimetric measurement of 5-hydroxyindoles produced from tryptophan, was insufficiently sensitive to detect enzyme activity in small cortical samples. The adequacy for this purpose of a radiometric assay described by Bensinger et al (126) was investigated. The principle of the method is as follows:

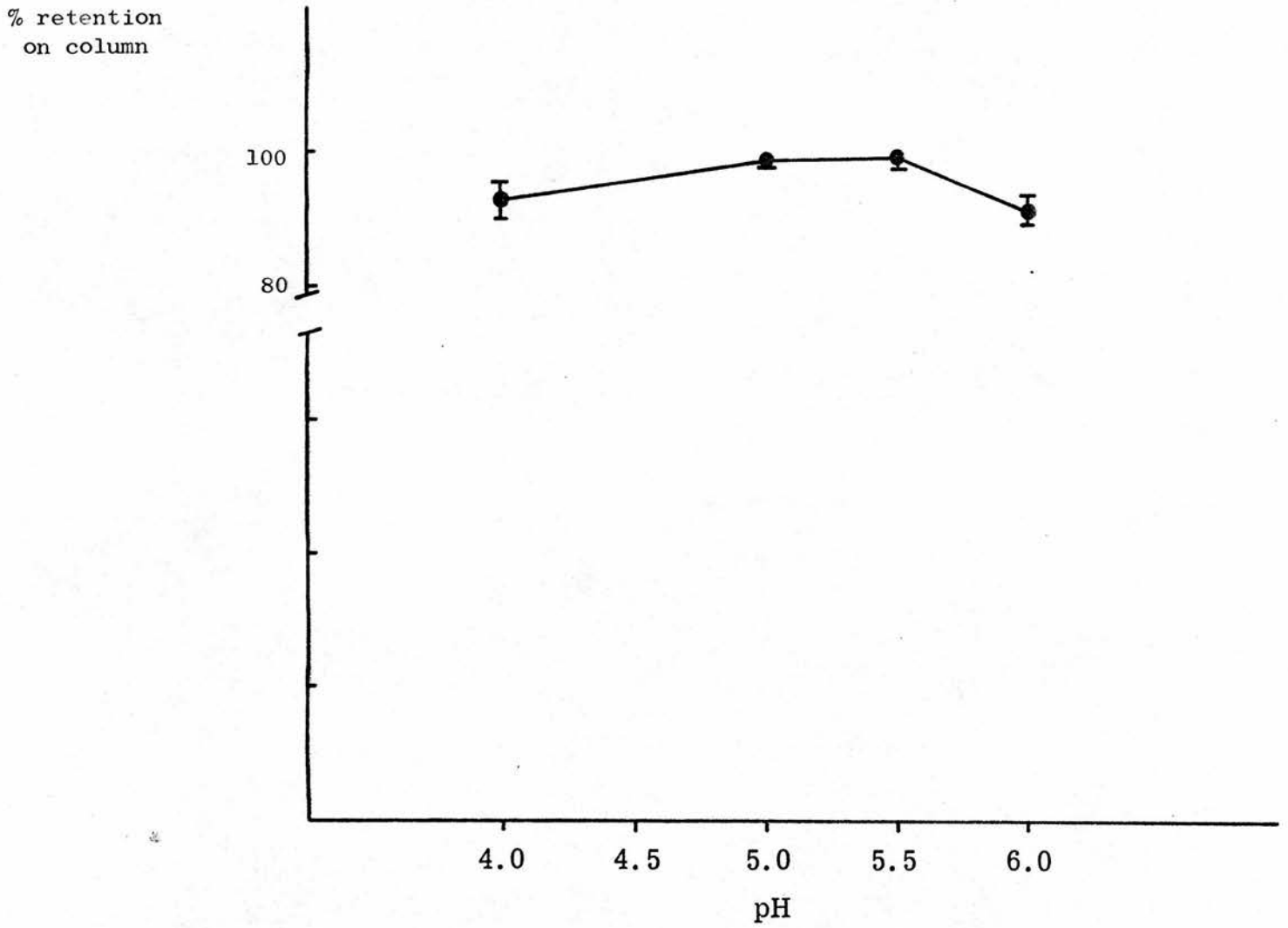
When (5-^{3}H)-tryptophan is hydroxylated at the 5-position, the ^{3}H is shifted to the 4-position (Guroff et al 573), where it exchanges readily with H^+ in acidic solutions. The amount of 5-hydroxylation of (5-^{3}H)-tryptophan can therefore be measured by the formation of tritiated water. The method of Bensinger et al (126) separates the tritiated water from unused (^{3}H)-tryptophan substrate by passing the incubation mixture through an ion-exchange resin which retains the labelled tryptophan, but not the (^{3}H)-water. The ion-exchange resin used was Dowex 50 x 8 (H^+); Bensinger et al found that this resin effectively absorbed tryptophan, and also tryptamine and melatonin. They also found that (4-^{3}H)-melatonin did not release enough (^{3}H) on acidification to bias the results of the assay, and therefore felt confident enough to state that when tryptophan hydroxylase activity was assayed using this method the tritiated water formed was a product only of the 4-^{3}H-5-hydroxyindole metabolites formed from 5-^{3}H-tryptophan. The following preliminary experiment was carried out to examine the method, using the experimental conditions detailed by Bensinger et al (126). 50 pmol of L-(5-^{3}H)-tryptophan, containing 1 μCi radioactivity (L-(5^{3}H)-tryptophan, 22 Ci/mmol, Amersham) was added to 500 μl 0.1 mM L-tryptophan in

distilled water. This solution of labelled and cold tryptophan contained the same amount of the substrate as used by Bensinger in subsequent enzyme assays. The solution was acidified by the addition of 50 µl 10N HCl and incubated for 15 min. at room temperature to permit (^3H)-(H^+) exchange. The solution was then passed through a 6 x 0.4 cm dia. column of Dowex 50 x 8 (H^+) resin, made up in a Pasteur pipette. The resin was washed before use with 20 ml 0.1 M HCl. After the sample had passed into the column, the column was washed with 2 x 4.00 ml 0.1 M HCl, the combined effluents being collected in a scintillation vial. 10 ml of Triton scintillant was added to the vial and samples were counted for 10 min. (external ratio mode), in a Hewlett-Packard scintillation counter.

As all (^3H) in the solution was theoretically in the 5-position of the labelled tryptophan, these column effluents should have contained little radioactivity since 5-^3H does not readily exchange with H^+, and both labelled and unlabelled tryptophan should have been retained on the columns. However, the effluents when counted were found to contain 1.0-1.5% (1.5×10^4 - 2.0×10^4 counts/min.) of the amount of the (^3H) applied to the column. To determine whether these counts were due to incomplete retention of tryptophan on the column or to the presence of either (^3H) water or L-(4-^3H)-tryptophan in the sample from Amersham, the initial acidification and incubation of solutions of labelled L-tryptophan were repeated, but after incubation the pH of the solution was altered using 0.5M NaOH to discover the optimal conditions for retention. The pH of the wash was altered to match, using different Tris (hydroxymethyl) methylamine buffers, 25 mM. The optimal pH range appeared to be between 5.0-5.5, when column

Fig. 8

Retention of tryptophan on a cation exchange resin (Dowex 50 x 8 H^+) at different pH values



1. pH values indicated refer to the pH the incubation solution was finally adjusted to, and also to the pH of the buffer solution used to wash the columns (Methods, BII).
2. Figures are given as $\bar{x} \pm SD'$, and all points refer to groups of 6 replicate samples.

retention was of the order of 99.14%. The (presumably) optimal results published by Bensinger et al are an order better than this, indicating a column retention of 99.92% efficiency; it seemed that the high reagent blank values obtained here could have been due to incomplete column retention and/or the presence of exchangeable ^{3}H in the sample from Amersham. This latter factor is probably involved to some extent as Bensinger reports some degree of non-enzymatic exchange of (H^{+}) with the (5-^{3}H). The blank values were considered unacceptably high for the proposed enzyme assays and the method was therefore not pursued further. A preliminary purification of the ^{3}H-tryptophan by ion exchange chromatography would have resulted in excessive dilution of the labelled compound.

III. Estimation of tyrosine-3-hydroxylase (TOH) activity in rat brain

The activity of the enzyme tyrosine 3-hydroxylase (L-tyrosine tetrahydrobiopteridine: O_2 EC 1.14.16.2) was estimated in samples of rat brain using the method of Hendry and Iverson (353).

The basis of the reaction is as follows: an extract of brain is incubated with ^{3}H-labelled tyrosine in the presence of an inhibitor of aromatic amino acid decarboxylase. At the end of the incubation period the amount of tritiated 3:4-dihydroxyphenylalanine (Dopa) formed is measured. This is proportional to the degree of tyrosine hydroxylase activity of the tissue extract. The incubation mixture containing both ^{3}H-Dopa and ^{3}H-tyrosine is passed through an alumina column. The tyrosine does not adsorb to the alumina and is washed out with a dilute buffer. The Dopa however is retained until eluted by an acid wash, a sample of which is then taken for ^{3}H counting.

^{3}H-2:3 side chain L-tyrosine (8 µg/ml, sp.act. 20 Ci/mmol, Amersham) was purified before use by incubation for 30 min. with alumina, to remove any ^{3}H-labelled catechol compounds that may have been present as impurities. This procedure was carried out at 0°C. 100 µl of the (^{3}H) L-tyrosine solution was added to a mixture of 100 µl of alumina slurry ('Neutral' grade, alumina Woelne, Eschwege:10 mg/100 µl distilled water) and 200 µl 5mM Tris (Tris-hydroxymethyl methylamine) buffer, pH 8.6. This mixture was shaken continuously for 30 min. using a mechanical shaking apparatus with sufficient force to keep the alumina in suspension. It was then centrifuged at 12,000 g for 1 min. The supernatant was used as the purified tyrosine substrate in the tyrosine hydroxylase assays. To produce enough substrate solution for 1 enzyme incubation 5 µl of the purified tyrosine solution was added to 5 µl of 0.2 M sodium phosphate buffer pH 6.0, containing 25 nmol tetrahydrobiopterin (Roche), 6.25 mmol mercaptoethanol, and 3.05 nmol 3-hydroxybenzyloxyamine (NSD-1055, Smith and Nephew) as the decarboxylase inhibitor. This was a concentration of NSD-1055 that had previously been found to effect a complete inhibition of aromatic amino acid decarboxylase activity, as measured using the method of Fonnum *et al*. (377). The final substrate solution, vol. 10 µl, contained 66 pmol (1 µCi) ^{3}H-2,3- side-chain L-tyrosine. To this was added an equal volume (10 µl) of tissue extract, prepared as described in the methods section A pt.III. The reaction was allowed to proceed for 20 min. at 37°C in a 1.5 ml stoppered Eppendorf reagent tube, and terminated by adding 200 µl 1 M perchloric acid, containing 1 µg L-Dopa as a carrier. The acid solution was then brought to approximately pH 8 by the addition of 4.0 ml of a solution made up of 0.1 M Tris-(hydroxymethyl) methylamine, 0.005 M Na Edetate, and 0.075 M

NaOH. The solution was then passed through a 5 x 0.25 cm (dia.) column of 'neutral' alumina, which had been previously washed through with 15 ml of 5 mM Tris buffer, pH 8.6. The unabsorbed unreacted ^{3}H-tyrosine was washed out of the column by the passage of 40 ml 5 mM Tris buffer pH 8.6, over 10-15 min. The ^{3}H-Dopa was subsequently eluted from the column using 3.0 ml 1.0 M-acetic acid, and a sample (1.0 ml) of the 3.0 ml eluate was added to 10 ml Triton scintillant (made up as specified in 'Materials'). The radioactivity of the samples was estimated in a Hewlett-Packard scintillation counter, (external ratio mode) the number of counts over 10 min. being recorded. The recovery of dopa through the whole procedure was measured as follows. 0.05 nmol ^{14}C-L-Dopa was added to a tissue homogenate (containing no ^{3}H-tyrosine) and then processed through the method, and the same amount of ^{14}C-Dopa was added directly to the effluent of a duplicate tissue sample processed through the method. The ratio of counts thus obtained gave a figure of around 75%. (Actual data, 73% $\pm$ 6.6% (10)). Tissue blanks were prepared in parallel with the samples of brain tissue, prepared by adding the perchloric acid to the tissue extract before adding the substrate solution. They gave counts of between 500-1,000 cpm. Nil values were assigned to all tissue samples with tyrosine hydroxylase activity less than 0.12 nmol dopa produced/g protein/h, or less than double the counts produced by tissue blanks.

A sub-saturating concentration of tyrosine was used (8 μM), as it has been found that, probably because of certain impurities typically present in the tyrosine, and a degree of non-enzymatic tyrosine hydroxylation, the sensitivity of the assay can best be increased by

Fig. 9

Tyrosine hydroxylase activity in cortical homogenates as a function of pH of incubation

Enzyme activity expressed as percentage of mean activity at pH 6.5 (4.53 ± 0.16 (4) n.mol. Dopa produced/g protein/h.). Vertical bars show standard deviation of 4 replicate samples at each pH value.

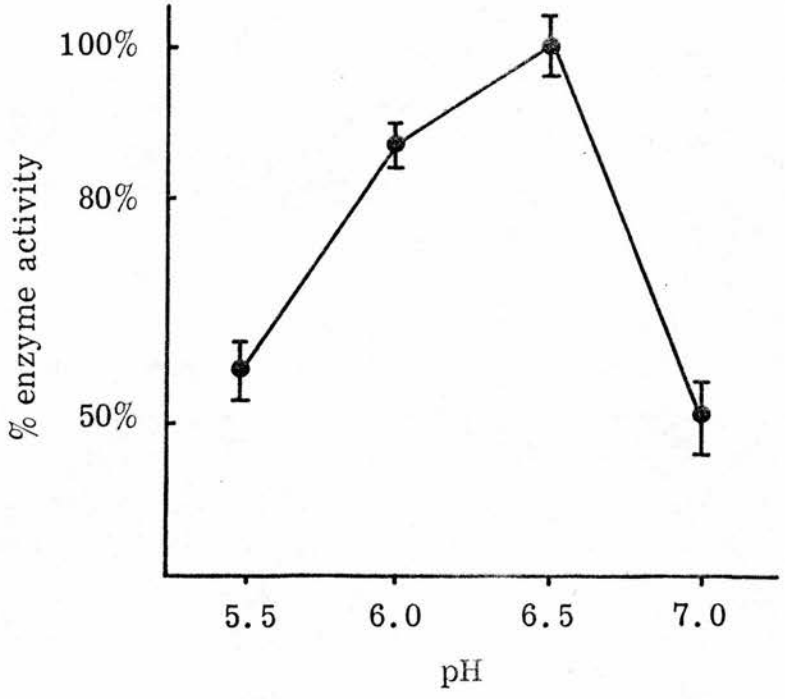
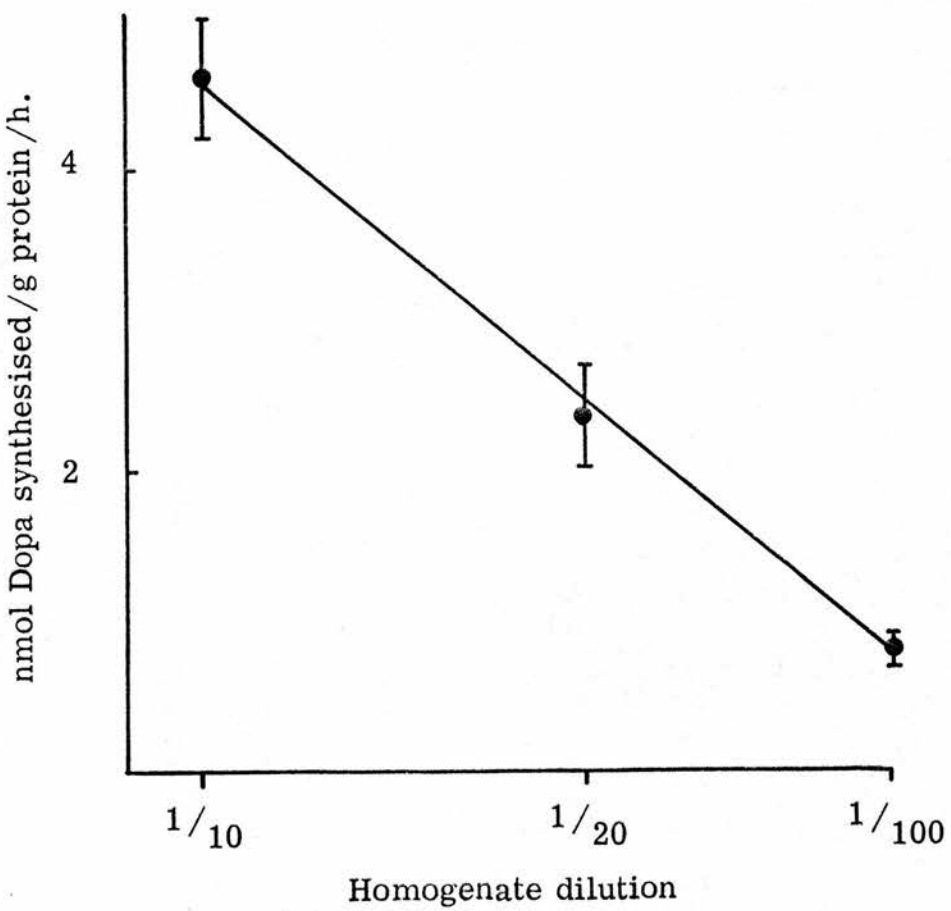


Fig. 10

Linearity of tyrosine hydroxylase activity in relation to enzyme concentration in cortical homogenates



1. A fixed volume of each homogenate was added to a standard incubation mix.
2. 1/10, 1/20 etc. refers to 1mg tissue in 10, 20 etc. µls homogenising buffer.
3. All points on graph refer to $\bar{x} \pm$ SD' for groups of 8 replicate samples

reducing tissue blank values (353). No significant loss of enzyme activity was found over periods of up to 25 days when the brain tissue was stored in liquid nitrogen: control groups of rats, the brains of which were stored for different lengths of time, showed no greater variation in levels of enzyme activity than groups of freshly killed rats. The validity of the enzyme assay as described was checked by establishing a linear relationship between enzyme activity and the concentration of the tissue homogenate (Fig. 9), and by establishing that the pH profile of the enzyme matched already existing data (Fig. 10).

IV Estimation of catechol-O-methyltransferase (COMT) activity in rat brain

The method based on that of Axelrod and Tomchick (351) was as follows, An extract of brain was incubated with a suitable substrate for O-methylation, in this case 3.4-dihydroxyphenylethyleneglycol (DOPEG), in the presence of a ^{14}C-labelled methyl donor, S-adenosyl-L-methyl-^{14}C-methionine (SAM). The amount of methylated substrate produced in a standard time was a measure of the COMT activity in the tissue extract. The ^{14}C-methyl labelled metabolite, 4 hydroxy-3-methoxyphenylethylene glycol (HMPG) was separated from the unused ^{14}C-SAM, by a process of phase partitioning, and a sample of the phase containing the HMPG was then taken for counting of the radioactivity as a measure of the amount of metabolite formed.

Stock solutions were prepared in distilled water immediately before beginning the assays. The contained DOPEG (2 mg/ml), $MgCl_2$, CH_2O (5 mg/ml), 0.5 M NaH_2PO_4/K_2HPO_4 buffer pH 7.8, and S-adenosyl-L-(methyl-C^{14}) methionine, 125 nmol/ml, S.A. 40 mCi/mmol, (Amersham) - stock solution.

The incubations were carried out in 1.5 ml stoppered polycarbonate reaction tubes. The total volume of each incubation (54 µl), was made up of 10 µl DOPEG solution, 20 µl $MgCl_2$ solution, 10 µl Na/K phosphate buffer, 10 µl of a ten-fold dilution in water of the stock SAM solution and 4 µl of a brain extract prepared as previously described (Methods A III). This reaction mixture was incubated at 37°C for 60 min., when the reaction was stopped by the addition of 1 drop conc. HCl. Solid sodium chloride was then added to saturation followed by 500 µl water-saturated ethyl acetate (chromatography grade). The reaction tubes were shaken vigorously by hand for 5 min. and then centrifuged for 2 min. at 12,000 *g* in an Eppendorf centrifuge. A sample, 400 µl, of the organic phase containing the HMPG, was added to 200 µl 0.1 M HCl in a 1 ml polycarbonate reaction tube and the mixture shaken and centrifuged as before; this washing procedure was included in the method to ensure complete removal of the ^{14}C-SAM in an attempt to lower tissue blank values. A sample, 250 µl, was taken from the upper organic phase and added to a scintillation vial containing 10 ml Triton scintillant. All counting was done on a Hewlett-Packard scintillation counter for 10 min. external mode. Tissue blanks, prepared with every series of assays, were of the order of 200-500 c.p.m. and represented from 1%-5% of the range of control enzyme values. They were prepared by adding the conc. HCl to the brain extract before the incubation mixture, followed by the otherwise normal assay procedure. 'Nil' values for the enzyme activity were assigned to samples of tissue with COMT activity of less than 14 nmol HMPG formed/g protein/h, equivalent to twice the 'tissue blank' counts. The validity of the enzyme assay as described was checked by establishing a linear

Fig. 11

Catechol-O-methyl transferase activity in cortical homogenates as a function of pH of incubation

Enzyme activity expressed as percentage of mean activity at pH 8.0 (371.5 ± 23.97 (6) n.mol. HMPG formed/g protein/h.). Vertical bars show standard deviation of 6 replicate samples at each pH value.

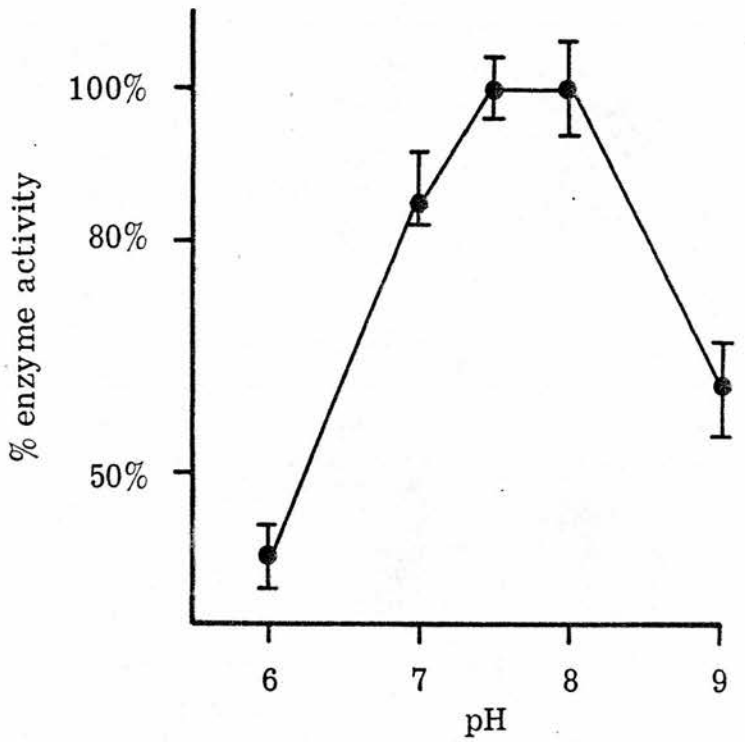
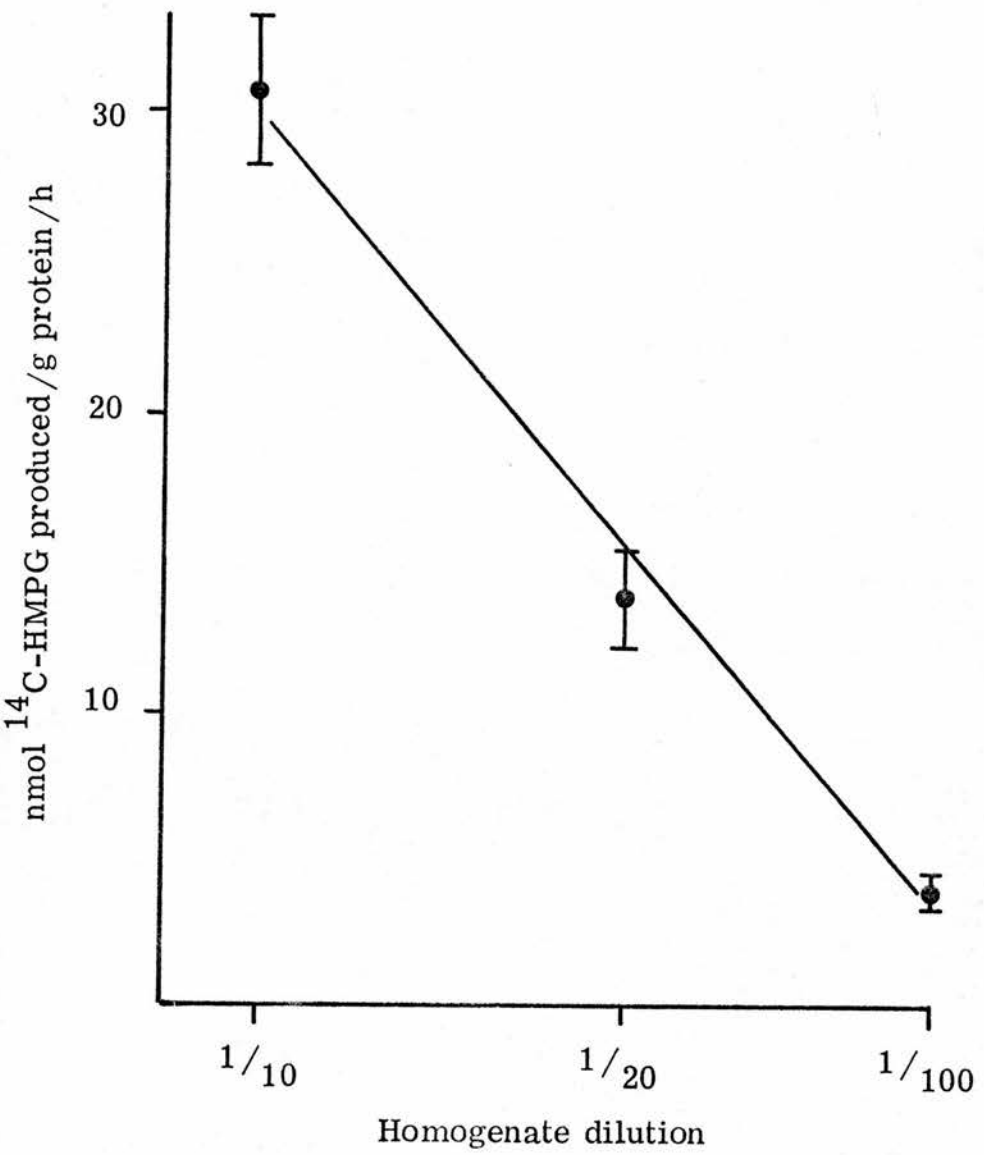


Fig. 12

Linearity of COMT activity in relation to enzyme concentration in cortical homogenates



ked volume of each homogenate was added to a standard incubation mix.
1/20 etc. refers to 1mg tissue in 10, 20 etc. µls homogenising buffer.
points on graph refer to $\bar{x} \pm$ SD' for groups of 8 replicate samples.

relationship between enzyme activity and the concentration of the tissue homogenate (Fig. 11), and by establishing that the pH profile of the enzyme matched already published data (Fig. 12).

V. Estimation of monoamine oxidase activity in rat brain

The method used was an unpublished modification by Emson of the method of McCaman and McCaman (352).

The basis of the assay is as follows. An extract of brain is incubated with ^{14}C-tyramine, and after a standard incubation period the unused substrate is removed into an organic phase. The ^{14}C-labelled metabolite, p-hydroxyphenylacetic acid, is measured in a sample of the aqueous phase to give an index of the amount of monoamine oxidase activity. 1 mg 1'-^{14}C-tyramine hydrochloride in 10 µl 0.1N HCl (317 µCi/mg, 55 mCi/mmol) was made up in 1.0 ml of distilled water containing 1 mg ascorbic acid. Equal volumes of this and 0.4 M sodium phosphate buffer pH 7.8 constituted the reaction medium.

2 µl of the reaction medium containing 4.3 x 10^8 c.p.m. was added to 2 µl of the brain extract (prepared as in AIII) in glass micro-tubes, and the mixture was incubated for 15 min. at 37°C. The reaction was stopped by placing the micro-tubes in ice-water. The contents of each tube was washed out into a 10 ml test-tube with 2.0 ml 10 mM phosphate buffer pH 7.0. To this solution was added 1 ml of a reagent consisting of 15 mg sodium tetraphenylborate/ml ethyl butyl ketone. The unused tyramine substrate was extracted into the organic phase by shaking gently the mixture in the stoppered glass tube for 5 min., followed by centrifuging at 2,000 g for 5 min. A 1 ml Oxford pipette with disposable tip was used to take a 1 ml sample

Fig. 13

Monoamine oxidase activity in cortical homogenates as a function of pH of incubation

Enzyme activity expressed as percentage of mean activity at pH 9 (35.06 ± 2.56 (9) μ.mol. tyramine oxidised/g protein/h.). Vertical bars show standard deviation of 9 replicate samples at each pH value.

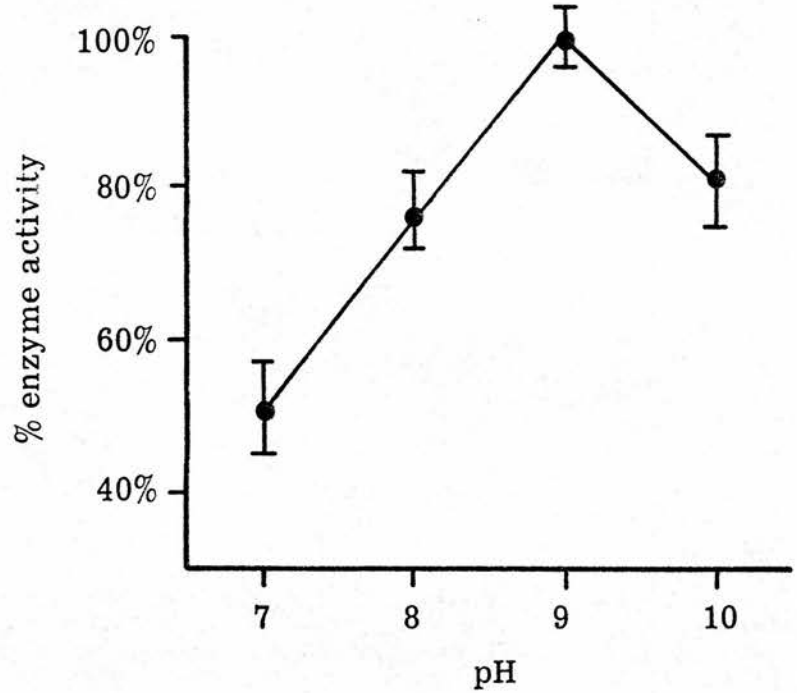
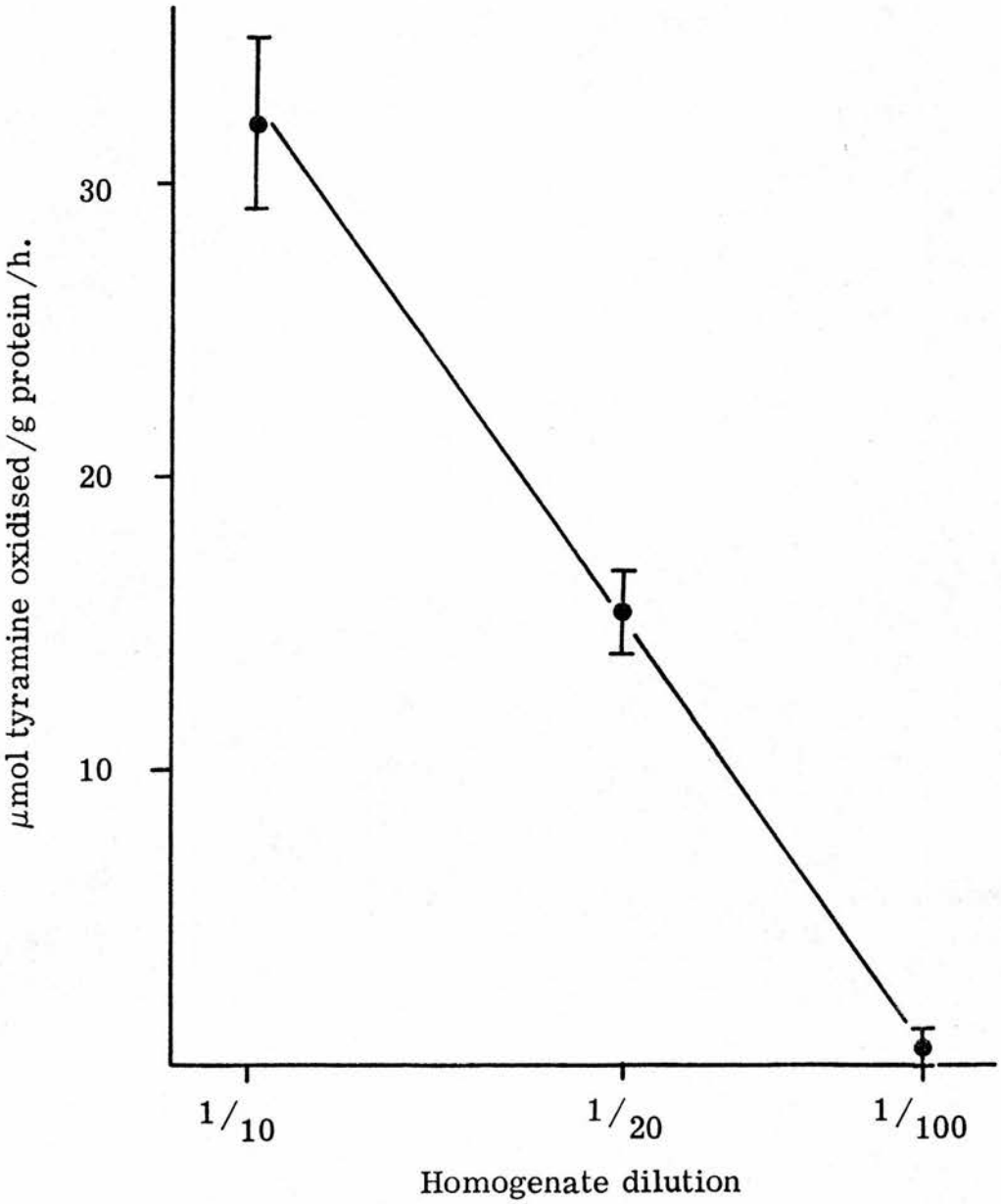


Fig. 14

Linearity of MAO activity in relation to enzyme concentration in cortical homogenates



fixed volume of each homogenate was added to a standard incubation mix.
'10, 1/20 etc. refers to 1mg in 10, 20 etc. µls homogenising buffer.
.l points on graph refer to $\bar{x} \pm SD$' for groups of 9 replicate samples.

from the lower aqueous phase. As the tip of the pipette was lowered through the upper organic phase, air was gently expelled from the pipette to prevent any of the organic phase from entering the tip. The sample of the aqueous phase was then added to 10.0 ml Triton scintillant and counted in a Hewlett-Packard scintillation counter, for 10 min., external mode. Tissue blanks were prepared by adding brain extract to incubation medium as above, but leaving the mixture in ice-water for 15 min. before processing as for other samples. The tissue blanks gave counts so low (300-600 cpm) as to suggest a nearly total transfer of unused tyramine to the organic phase - that is, better than 99.9%. Nil values were assigned to samples with MAO activity less than 0.4 µmol tyramine oxidised/g protein/h, equivalent to sample counts less than twice the 'tissue blank' counts. The validity of the enzyme assay as described was checked by establishing a linear relationship between enzyme activity and the concentration of the tissue homogenate, and by showing that the pH profile of the enzyme matched already published data (Figs. 13 & 14).

C. ASSAYS OF NEUROTRANSMITTERS, PRECURSORS AND METABOLITES

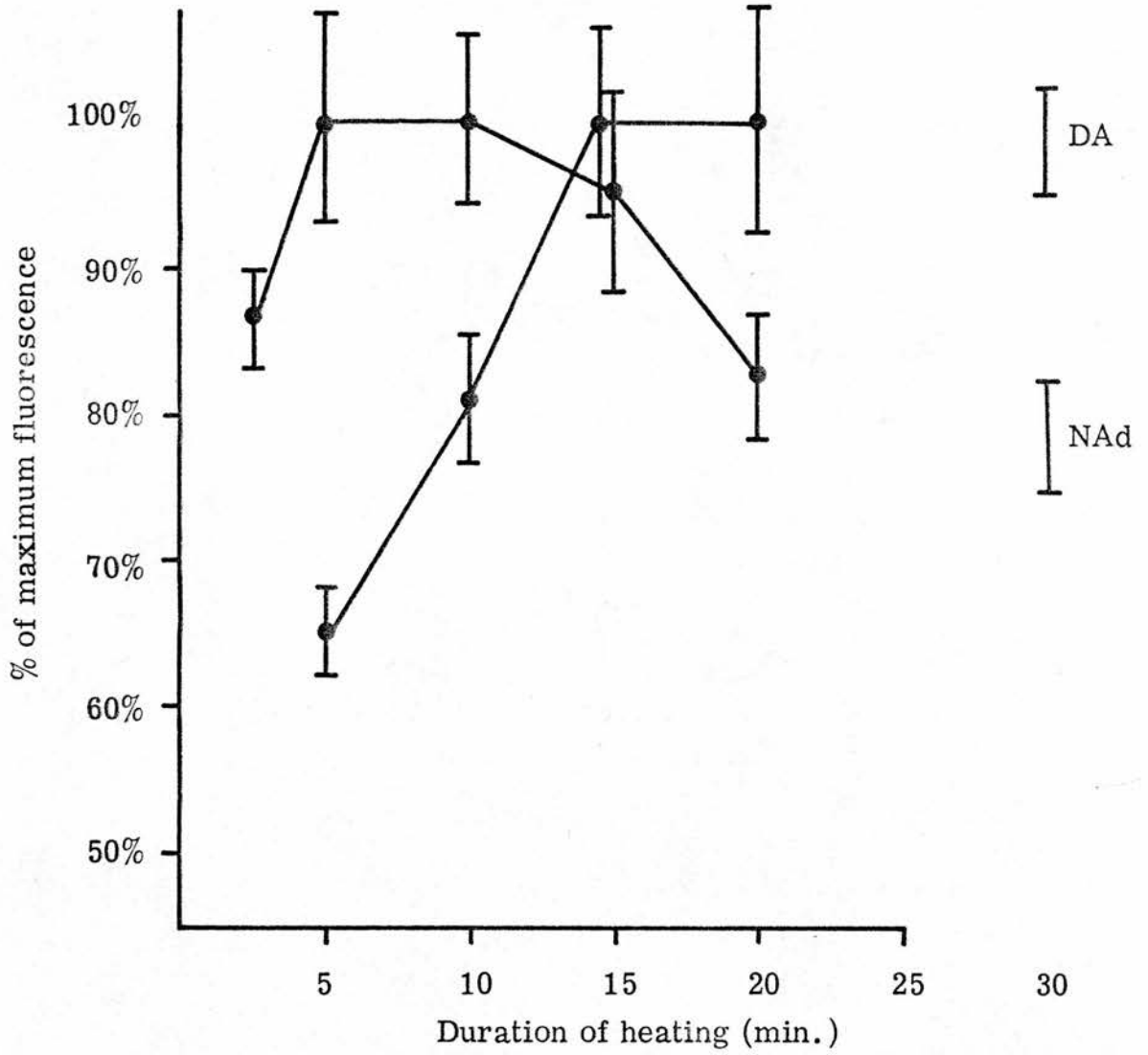
I. Estimation of noradrenaline (NAd) and dopamine (DA) in rat brain

This procedure was based on the method of Schlumpf *et al* (24). All the following steps were carried out at 0-4°C. Sections of brain, 20-25 mg, were weighed and homogenised (1 mg in 10 µl) in HCl-butanol (850 µl conc. HCl, BDH 'Aristar' in 1 litre n-butanol, spectroscopy grade), for 1 min. in an all-glass homogeniser, working capacity 500 µl. The homogenate was transferred to a 1.5 ml Eppendorf reagent tube and centrifuged for 3 min. at 12,000 g. A portion, 160 µl, of the upper organic phase was removed and added

Fig. 15

Development of a dual fluoroassay of NAd and DA in cortical tissue

All figures refer to the percentage of the maximum fluorescence produced by either amine at the optimum incubation time. Vertical bars refer to the standard deviations of groups of 6 replicate samples. (Methods CI).



to 400 µl heptane (BDH, spectroscopy grade), and 50 µl 0.1 M HCl, in a 1.5 ml polycarbonate reagent tube. The mixture was shaken vigorously by hand for 10 min. and then centrifuged as before to separate the two phases. The upper organic phase was carefully removed, and a portion of the aqueous phase (40 µl) taken for either NAd/DA estimation (below) or for 5-HT estimation (CV).

To 40 µl of the aqueous phase in a 1.5 ml polycarbonate reaction tube was added 10 µl 0.4M HCl and 20 µl EDTA/sodium acetate (4M sodium acetate, 5 mM Na Edetate, pH 6.9), followed by 20 µl iodine solution (0.1M in ethanol) for oxidation. The reaction was stopped after 120 sec. by the addition of 20 µl of a solution of sodium sulphite in NaOH (0.5g $Na_2SO_3.7H_2O$ in 2ml H_2O + 18 ml 5M NaOH), and after a further 90 sec. 20 µl 10 M acetic acid was added. The solution was heated, in the sealed reaction tube, at 100°C for 13 min., conditions which were found to be the optimal compromise for the simultaneous development of both the NAd and the DA fluorophore (Fig. 15). Samples were then rapidly cooled to room temperature, and each transferred to a microcuvette (capacity 200 µl) for fluorimetry in a Perkin-Elmer spectrofluorimeter, MPF-2A. The excitation (and occasionally the emission) spectrum was recorded from each sample: excitation and emission wavelength maxima were respectively 395nm and 485nm for the NAd fluorphore and 330nm and 375nm for that from DA. The excitation spectrum was recorded between λ = 435 nm and λ = 345 nm for NAd, and between λ = 280nm and λ = 380nm for DA. Various primary and secondary filters were tried, but none were found to have any significant effect on the method. The slit widths of both excitation and emission monochromators were generally set at 5 nm.

These figures all refer to uncorrected instrument values.

Using the above conditions the excitation spectra showed no evidence of any cross-interference. Flurophore-containing solutions derived from pure samples of NAd gave nil readings when the recording conditions were set for DA, and vice versa. Quantification was by comparison with standard curves obtained by processing aqueous solutions of the two amines through the method, and further corrected using a method recovery estimate, obtained as follows. Identical amounts of NAd and DA (50 ng of each) were added to a control homogenate and then processed through the method. The same amounts were also added directly to the final 40 µl acidic phase from a duplicate tissue sample. When the fluorescence readings due to endogenous amines had been subtracted from each 'total' fluorescence value (including endogenous and the exogenous amine) this gave a figure for recovery of the amines through the method of 51% $\pm$ 7% (DA) and 42% $\pm$ 5.5% (NAd) from a series of 8 duplicate samples. This correction was used when referring fluorescence readings in tissue samples to the standard curves. The amount of each amine used (50 ng) had previously been found to occur within the linear amine concentration/ fluorescence intensity range.

The sensitivity of the fluoroassay (arbitrarily assessed as that amount of the amine which produces a fluorescence equal in size to that of the tissue blank) was approximately 15 ng of either amine; the degree of fluorescence attributable to this amount of exogenous amine, when added to a control homogenate of 25 mg cortex, was approximately double that of 'tissue blanks'. 'Tissue blanks' were obtained by adding the acetic acid to the amine-containing extract before the sodium sulphite, and adding the iodine last of all.

II. Estimation of homovanillic acid (HVA) in rat brain

The method is based on those of Walter and Eccleston (562) and of Roos (211). The extraction procedure described in this method is also suitable for use in conjunction with the fluoroassay of 5HIAA, which is extracted like the HVA into the 0.15 M sodium borate buffer (below). This method was used for one series of 5HIAA assays mainly to verify data generated using a different extraction technique (Methods C, section V). The particular analytic method employed is noted with each series of estimates cited in the results section.

Samples of brain tissue were processed as follows. After weighing, the samples (20-35 mg) were homogenised at below 5°C in 2.5 ml 0.4 M-perchloric acid in an all-glass apparatus. The homogenate was transferred to a 5 ml glass test-tube with one wash of 0.5 ml 0.4 M-perchloric acid. The combined homogenate and wash was then centrifuged at 10,000 g for 15 min. at 0°C. The supernatant was removed and adjusted to pH 4-5 (glass electrode) with 3 M-KOH. After 15 min. at 5°C, to obtain maximal precipitation of the potassium perchlorate, the sample was centrifuged at 3,000g for 10 min, and the supernatant was transferred to a C24 ml glass test-tube containing 50 µl of a freshly prepared 2 mg/ml solution of ascorbic acid. The sample was adjusted to pH 2.5 (glass electrode) using 2 N HCl, then saturated with NaCl. The phenolic acids were extracted into butyl acetate (B.D.H. redistilled and water-saturated) by shaking the sample twice for 5 min, eachtime with 10 ml of the solvent. The butyl acetate extracts, 9 ml and 10 ml respectively, were combined after separation from the aqueous phase by centrifugation at 2,000 g for 5 min.

The phenolic acids were returned to aqueous solution by shaking the pooled butyl acetate extract for 5 min. with 1.4 ml 0.15 M sodium borate buffer, pH 8.6. After centrifugation at 2,000 g for 5 min, the upper organic layer was aspirated off and a 500 µl portion of the aqueous phase was placed in a 1.5 ml poly reaction tube containing 200 µl of an alkaline potassium ferricyanide reagent. This reagent made up immediately before use, consisted of 25 ml 9 M ammonium hydroxide solution to which was added 100 µl of a stock solution of potassium ferricyanide of 11 mg/ml. After 4.0 min. the oxidation was terminated by the addition of 50 µl of a freshly prepared 1.75 mg/ml solution of cysteine.

The fluorophore thus obtained from HVA was stable for several hours, and has a maximum activation wavelength of 320 nm and a maximum fluorescence wavelength of 425 nm. (uncorrected instrument settings) For each sample, activation spectra were recorded from 260 nm to 390 nm, using a Perkin-Elmer spectrofluorimeter model MPF-2A with the slit widths on both monochromators set at 8 nm, and the emission monochromator set at 425 nm. The fluorescence intensity at peak fluorescence (λ = 320 nm) was used as a measure of the fluorophore content, in relation to those of aqueous standards (10-200 ng), processed through the method. These amounts of HVA all fell within the linear range of the HVA concentration/fluorescence intensity curve.

Tissue blanks, obtained by mixing the fluorophore-developing reagents (the cysteine and ferricyanide solutions) together before adding them to the HVA-containing fraction, were as low as reagent blanks. The recovery of HVA through the method was derived as follows: 40 ng of HVA was added to a control homogenate of 25 mg

cortex, and also directly to the final 500 µl aqueous sample derived from a duplicate cortical sample. The recovery of HVA through the method was derived from the ratio of the fluorescence attributable to the exogenous HVA when added either to the homogenate or to the 500 µl aqueous sample. The figure was 25.8% ± 7.25 (8). The sensitivity of the assay, arbitrarily assessed as that amount of exogenous HVA, which when added to a control homogenate of 25 mg brain and processed through the method produced a fluorescence equal in size to that of a tissue blank, was of the order of 40 ng.

III. Estimation of 4-hydroxy-3-methoxyphenylethylene glycol (HMPG) in rat brain

The method of estimation was based on that described by Walter and Eccleston (562). Weighed samples of brain (10-20 mg) were homogenised at 5°C in 2.5 ml 0.4 M perchloric acid, in an all-glass homogeniser. The homogenate was transferred to a 5 ml centrifuge tube with washing with a further 0.5 ml 0.4 M perchloric acid, and the combined homogenate and washings centrifuged at 10,000 g for 15 min. The pH of the supernatant was adjusted to between pH 4-5 (glass electrode) with 5 M-KOH and the sample was kept for a further 15 min. at 5°C to obtain maximum precipitation of the potassium perchlorate. The sample was centrifuged at 3,000 g for 10 min. at 5°C, and portions of the clear supernatant were taken for HMPG assay.

HMPG exists in the brain both as the free compound and as its sulphate ester (Schanberg et al, 578). The brain extract was treated therefore to hydrolyse the sulphate conjugate prior to assaying total HMPG content. A 3 ml sample of the clear supernatant from the homogenisation stage was adjusted to pH 5.0 using 200 µl 1.0 M sodium acetate buffer pH 5.0, and incubated at 37°C for 16 h in

a stoppered 1.5 ml polycarbonate reaction tube with 100 µl of a 50 mg/ml solution of the sulphatase-containing enzyme preparation "Helicase" (Industrie Biologique Francais). The HMPG was extracted into ethyl acetate (Reeve Angel Scientific Ltd., C.T. grade) by shaking the sample twice for 5 min. each time, with 8.0 ml and then 5.0 ml of the solvent. The ethyl acetate extracts, 7.0 ml and 5.0 ml respectively, were combined in a 100 ml round-bottomed flask, after separation from the aqueous phase had been aided by centrifugation at 2,000 g for 5 min. and were reduced to a small volume (1-2 ml) by evaporation at 50°C under reduced pressure. The extract was transferred to a 5 mlglass test-tube and reduced to dryness at 56°C under a stream of dry N_2. The residue was dissolved in 0.4 ml distilled water, and the HMPG was acetylated (Sharman, (563)) by the addition of 50 µl redistilled acetic anhydride (Sigma) and 0.6 ml of a 16.5% solution of potassium bicarbonate (Walter, personal communication). The reaction was allowed to continue with occasional gentle shaking for 30 min. and the acetyl-HMPG extracted from the aqueous solution by shaking for 1 min. with 1.6 ml 1:2-dichloroethane (BDH laboratory reagent, redistilled). After centrifugation at 2,000 g for 5 min., 1.3 ml of the bottom organic layer was transferred to a 5 ml glass stoppered test-tube containing a small quantity of anhydrous sodium sulphate and shaken to remove residual traces of water. After centrifugation at 10,000 g for 2 min. the 1:2 dichloroethane extract was decanted into a 2 ml borosilicate test-tube and reduced to dryness under a stream of N_2, at 50°C. The acetyl-HMPG was converted to the trifluoroacetylated form (Bond, 564) by dissolving the residue in 0.6 ml of a mixture of 1 vol. redistilled trifluoracetic anhydride (Sigma) and 5 vol. ethyl acetate. The tube was stoppered and heated at 56°C for

15 min. The solution was then blown to dryness under a very gentle stream of N_2 at room temperature, and the residue dissolved in 0.2 ml ethyl acetate containing 30 ng/ml hexachlorocyclohexane as a marker to enable variations in final injection volume (into the GLC apparatus) to be allowed for (Sharman 563). As the injection volume could not be completely standardised, measuring the HMPG/hexachlorocyclohexane peak height ratio instead of HMPG peak height alone enabled samples to be compared directly. Analysis of the sample was carried out on a Perkin-Elmer 900 gas chromatograph with a 6 ft. column, internal diameter 4 mm. The stationary phase was 2.5% silicone gum rubber E301 on a support of Chromosorb GAW DMCS, 80-100 mesh. The carrier gas was an argon/methane mixture (90%/10% v/v) with a flow rate through the column of 40 ml/min. Detection was by means of ^{63}Ni electron capture detector. Polarising voltage was in the pulse mode with a negative amplitude of 50v and 1 m sec pulse duration. Instrument ovens were set at the following temperatures - injection, 270°C; column, 170°C; manifold, 270°C; electron capture detector, 280°C.

Under these conditions the retention time of trifluoroacetyl-HMPG (7 min) was half that of the hexachlorocyclohexane marker. Quantitative analysis was performed by comparison with a standard curve obtained by processing HMPG through the method, adding standard amounts (10-100 ng) of HMPG to duplicate samples of cortical homogenate. Recovery of HMPG through the extraction and derivative formation was found to be of the order of 30% (35.1/ $\pm$ 3.3 (10)), for 100 ng of added HMPG. The relationship between the amount of HMPG added to a standard homogenate of brain, and the subsequent peak height on the G.L.C. was found to be linear over the range 50-150 ng.

IV. Estimation of tryptophan in rat brain

This procedure was based on the method of Hess and Udenfriend (242) in which the fluorescent norharman, derived from tryptophan, is measured. Weighed samples of cortex (20-30 mg) were homogenised in distilled water, 10 µl/mg, at 100 r.p.m. for 2.5 min. After centrifugation at 10,000 g for 5 min. 1.00 µl portions of the supernatant were added to 1.0 ml trichloroacetic acid (50 g/l) in a 2.0 ml glass test-tube.

Ten min. after thorough mixing, the contents of the tubes were centrifuged for 2.5 min. at 2,000 g. 400 µl portions of the supernatant were removed and added to 100 µl 0.25 M-H_2SO_4 and 100 µl formaldehyde (40g/l) in 1.5 ml polycarbonate reaction tubes. The tubes were stoppered and heated to 96°C for 20 min., when 100 µl 1.2% w/v H_2O_2 was added. The hydrogen peroxide solution was made up immediately prior to use by diluting a stock 6% w/v BDH preparation. Heating was then continued for a further 20 min. After cooling in ice the reaction mixture was transferred to a cuvette in a Perkin-Elmer spectrofluorimeter, model MPF-2A for fluorimetry. Excitation scans were recorded from = 320 nm to = 420 nm with the emission monochromator set at = 450 nm (the wavelength of maximum fluorescence). Monochromator slit widths were both 10 nm. The figures quoted refer to uncorrected instrument settings. The sensitivity of the method was such that the fluorescence attributable to 15 ng L-tryptophan, added to a homogenate of 25 mg cortex and processed through the method, was at least double the fluorescence of "tissue blanks". The "tissue blanks" were obtained by adding distilled water in place of hydrogen peroxide to the tissue extracts.

Background fluorescence could be allowed for by measuring the norharman-specific fluorescence in terms of the distance 'h', as in Fig. 20. This procedure of course assumes that the baseline fluorescence does not alter over the wavelengths recorded, or, if there is an alteration, that this remains constant with every sample. Although this cannot be directly examined, the baseline fluorescence in the 'tissue blanks' was found to be stable over the critical wavelengths. Data were also corrected using a recovery factor obtained by comparing the fluorescence attributable to 50 ng L-tryptophan when added either to distilled water, or to a homogenate of 25 mg brain and processed through the method. This recovery factor was of the order of 80%. (Actual values as used in the assay of cortical tryptophan, 78% $\pm$ 7%, (10)).

V. Estimation of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic Acid (5-HIAA) in small areas of rat brain

The simultaneous assay of 5-HT and 5-HIAA in small areas of rat brain is currently generally carried out using one of two types of fluoroassay. The first of these two types is based on the measurement of the fluorescence with characteristics specific to the 5-hydroxyindoles which develops at acid pH, and the second type is based on the reaction between these compounds and o-phthal dialdehyde (OPT) with the resulting formation of highly fluorescent complexes (441).

Of the first type, the methods of Giacolone and Valzelli (438), Welch and Welch (439) and Jonson and Lewander (440) all required several hundred mg of tissue, necessitating therefore the pooling of samples from groups of rats. These methods have been superseded very recently by a fluorimetric method published by Schlumpf et al, (24),

utilising the extraction procedures originally described by Chang (238) and Maickel et al (239). This new method can assay the two compounds in pieces of tissue only 2-3 mg in weight. The OPT-based double assay of Maickel et al (442) was reported to be equally sensitive, and the two types of assay procedures were therefore examined so that the more reliable could be used for the later experiments.

All brain samples used were taken from rats killed between 10.30 and 11.30 am, to minimise the effect on the assays of the circadian rhythms of central levels of 5-HT and 5-HIAA (52). 20-30 mg samples of freshly removed or deep-frozen (in liquid N_2) rat cerebral cortex were homogenised in 10 volumes HCl/butanol (85 µl 37% HCl B.D.H. Aristar in 100 ml n-butanol, B.D.H. spectroscopy grade). The homogenisation was carried out in all-glass apparatus, 1.5 minutes at 2,000 rpm. The homogenate was then transferred to 1.5 ml polycarbonate reaction tubes and centrifuged for 2.5.min at 12,000g. A portion of the supernatant (160 µl) was added to 400 µl heptane (B.D.H. spectroscopy grade) and 50 µl 0.1M HCl in 1.5 ml polycarbonate reaction tubes. The tubes were then stoppered and shaken vigorously by hand for 10 min. The phases were separated by centrifugation for 2.5 min. at 12,000g and the lower aqueous phase was used for the estimation of 5-HT (see below). A 500 µl portion of the upper organic phase, containing the 5-HIAA, was added to 60 µl 0.15 M sodium borate buffer, pH 8.6. This two-phase mixture was shaken for 5 min. and then centrifuged as before for 2.5 min. at 12,000g. The upper organic phase was carefully aspirated off, and a 50 µl portion of the aqueous phase taken for 5-HIAA estimation. At this stage the remaining small volume of heptane/butanol was aspirated off from the

aqueous extract containing the 5-HT, and a 40 µl portion taken for the assay.

ASSAY BY THE HCl METHOD

To each 50 µl '5-HIAA' samples or 40 µl '5-HT' samples was added 20 µl concentrated HCl (B.D.H. Aristar) containing 0.5 mg/ml ascorbic acid, the solution being prepared immediately before use for a series of estimations. Each sample was mixed thoroughly and transferred immediately to a micro-cuvette in a Perkin-Elmer spectrofluorimeter model MPF-4. The fluorescence spectrum of the sample was recorded with the emission monochromator set at λ = 540 nm while the excitation light was continuously altered from λ = 240 nm to λ = 340 nm (excitation scans). Slit widths of 8 nm were used in both the excitation and the emission monochromators, and a filter (UV - 39) cutting off light below λ = ~360 nm was interposed in the emitted light beam between the micro-cuvette and the emission monochromator so that none of the activation light or harmonics thereof would reach the phototube. All quoted figures are uncorrected instrument settings. Emission scans were not used: one criticism of this method is that direct comparison between excitation and emission curves was not possible, as the emission scan did not show a definite peak at the appropriate 5-hydroxyindole wavelength. The appearance of a typical excitation spectrum for 5-HT or 5-HIAA is shown in Fig. 16. The excitation maximum occurs close to 300 nm, and all readings were corrected for background fluorescence by assaying the indoles in terms of the distance 'd'. This method assumes that baseline fluorescence remains unchanged over the wavelengths measured; in fact, there is a minor peak of fluorescence associated with the HCl at these wavelengths, as shown in reagent blanks, but this appears to make a fairly constant

contribution, and therefore cannot affect assay results. The fluorescence attributable to 10 ng of either 5-HT or 5-HIAA when added to a homogenate of 25 mg cortex and processed through the method was equal to or slightly greater than the fluorescence of a reagent blank. Whenever samples were processed, a series of aqueous standards were also assayed. This was done by shaking 25 µl distilled water containing known amounts of 5-HT or 5-HIAA (5-100 ng) with 250 µl HCl/butanol, and then treating the standards as the other samples. Recovery factors were also estimated on each occasion, comparing the fluorescence attributable to 100 ng of either 5-HT or 5-HIAA when added directly to HCl/ascorbic acid and when added to samples of brain homogenate. For the three major experiments involving the assay of the 5-hydroxyindoles, the recovery values from brain homogenates were as follows:

5-HT - 63%, 65%, 63%

5-HIAA - 84%, 75%, 82.5%

B. 5-HYDROXYINDOLE ESTIMATION BY FLUORIMETRY OF THE REACTION PRODUCT FORMED WITH O-PHTHALDIALDEHYDE (OPT)

This method, based on that of Maickel et al (442) was examined as a possibly more sensitive alternative to that described above. Both methods involve the same extraction procedure, beginning with the brain tissue being homogenised in 10 vol. HCl/butanol, and ending with 5-hydroxyindoles in aqueous solution (5-HT in 0.1 M HCl and 5-HIAA in 0.15 M sodium borate buffer respectively).

Samples were taken of the 5-HT-containing 0.1 M HCl phase (40 µl) and of the 5-HIAA-containing 0.15 M borate buffer (50 µl), and to either of these was added 50 µl of the OPT reagent (0.2 mg o-phthaldialdehyde in 1.0 ml conc. HCl). The fluorophore was developed by heating at

Fig. 17

5-HIAA estimation by OPT and native fluorescence methods

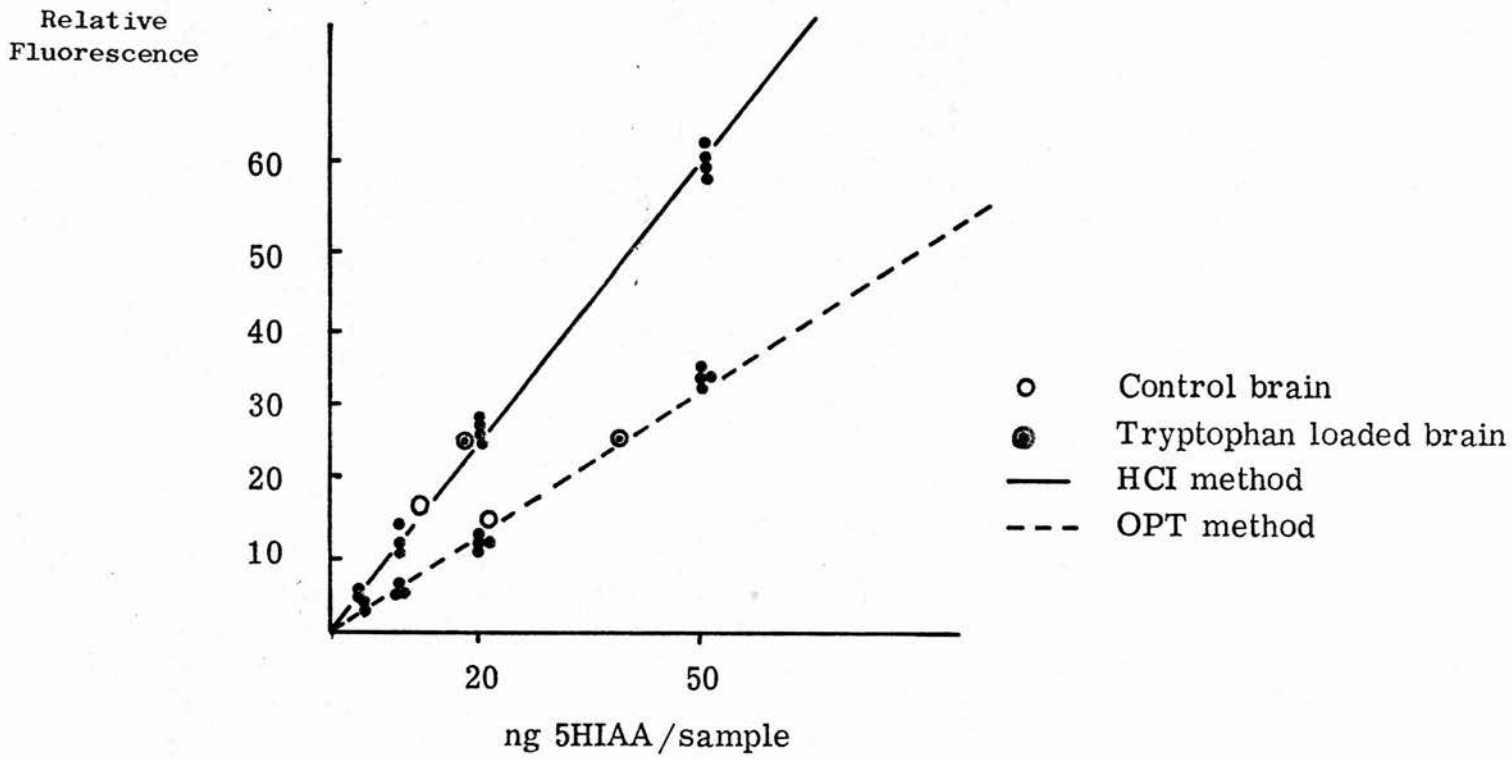
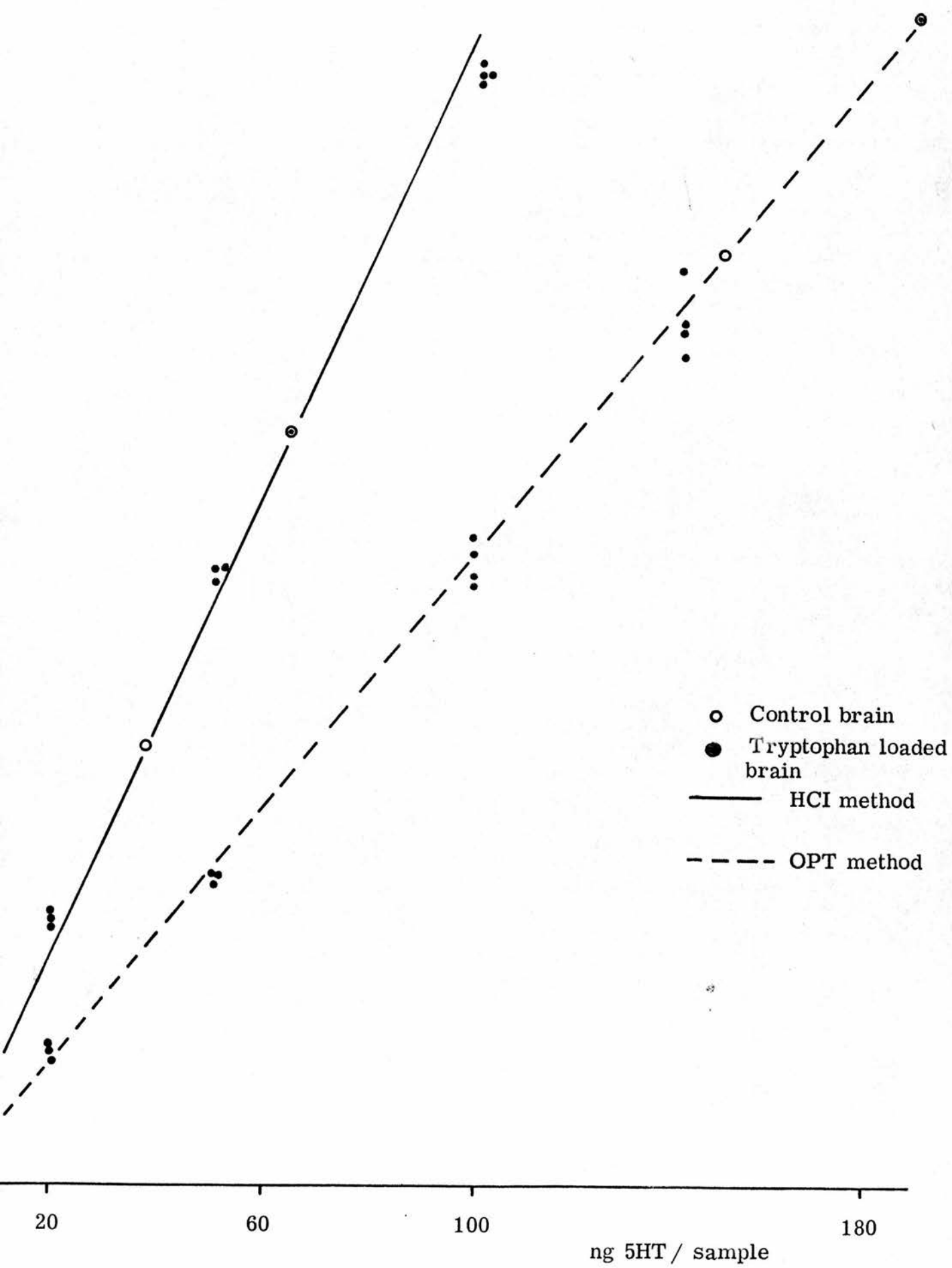


Fig. 18

5-HT estimation by OPT and native fluorescence methods



100°C for 10 min. in a stoppered 1.5 ml polycarbonate reaction tube. The samples were then rapidly cooled by immersion in ice-water. The 5-hydroxyindole-containing samples were transferred to a micro-cuvette (capacity 200 µl); and analysed in a spectrofluorimeter, the Perkin-Elmer model MPF-4A.

The excitation maximum occurred at λ = 360 nm, the emission maximum at λ = 470 nm. Excitation spectra were recorded from λ = 300 nm to λ = 420 nm and the slits of both monochromators were set at a width of 5 nm. A filter cutting off light below λ = 420nm was inserted in the light beam between the micro-cuvette and the emission monochromator. The quoted instrument settings are uncorrected. Quantification was by comparison with a series of aqueous standards containing 10-150 ng of 5-HT or 5-HIAA. Typically, the fluorescence attributable to 15 ng of either 5-hydroxyindole when added to a homogenate of 25 mg brain and processed through the method was approximately twice the fluorescence of "tissue blanks", which were obtained by processing control tissue samples through the method, but treating them finally with pure HCl only, containing no OPT.

To compare the two methods, duplicate samples from control cortical homogenates were processed by the two different methods (Fig. 17 & 18). In fact the HCl method gave results for the control brain samples that agreed very closely with published data, while the apparently greater 5-hydroxyindole concentrations as measured using the OPT method may have been due to the formation of OPT-fluorophores with other compounds (441, 442, 443). It was therefore decided to use the HCl method.

D. OTHER METHODS

Ia. Estimation of protein in rat brain samples

In order to avoid problems in the quantification of various biochemical parameters in brain tissue associated with possible alterations in the water content of the samples due to oedema, the concentrations of the variables have usually been recorded in terms of protein concentrations. Protein estimations were made on portions of the brain extracts used in the biochemical assays. The Waddell method of protein estimation was used (572) in preference to that of Lowry (584), as it was found (466) that cobalt salts interfered with the Folin phenol method of Lowry.

Samples of 50 µl were taken from the clear supernatant obtained from brain samples extracted as described in Methods A, part V. These were diluted 1 in 25 in sodium chloride solution, 9 g/l. Using a Pye Unicam spectrophotometer, model SP 500 series 2, the absorbance of each diluted extract was measured at 215 nm and 225 nm, and the latter value subtracted from the former; the value thus obtained was converted to a protein concentration by comparison with a standard curve, obtained by processing known amounts of bovine serum albumin (Sigma) through the method. Reagent blanks were obtained by using a saline dilution of the homogenising buffer used in the preparation of the brain extracts, as on p6a.

The rationale of this method is af follows. The absorption at these low wavelengths is due principally to the peptide bond - unlike the absorption maximum at λ = 280 nm which is due to aromatic amino acid moieties. The latter absorption factor obviously differs far more between proteins than the former. Waddell found that absorption concentration curves of different proteins at the lower wavelength are so similar that the same standard curve could be used

to calculate the concentration of either human serum albumin or globulin, without significant error. With this method Waddel found that in almost all biological specimens the concentration of protein is so high, relative to possible interfering materials, that the method is essentially specific. The presence of nucleic acids, which can seriously interfere with determination of protein when absorption at 280 nm is measured, does not affect this method.

Ib. Estimation of cerebral protein synthesis

The method used was based on that of Barondes and Cohen (595). 48 h. after the intracerebral injection of 20 µg acetoxycycloheximide in 10 µl Krebs' Ringer's solution into the frontal cortex, the experimental rats were given a subcutaneous injection of 1 µCi of a ^{14}C-labelled protein hydrolysate, made up in 0.5 ml 0.9% NaCl ([U-^{14}C] Protein hydrolysate, s.a. 45 mCi/milliatom of carbon, Amersham). One hour later, the rats were stunned, decapitated and the cortex from both cerebral hemispheres was removed and homogenised in 0.1 N NaOH, 1 ml/25 mg. 3.0 ml of cold 12% trichloroacetic acid (TCA) was added to 1.0 ml of the homogenate and this was then left for 10 min. at 0-4°C. The suspension was centrifuged at 10,000 *g* for 2.5 min. and aliquots of the supernatant (250 µl) were added to 10 ml Triton scintillant (TCA-soluble fraction). The protein-containing sediment was re-suspended and re-centrifuged twice in 10% TCA. After the last centrifugation the TCA was allowed to drain off and the insoluble pellet was solubilised in 1 ml 0.1 N NaOH. This was then also added to 10 ml Triton scintillant (TCA-insoluble fraction). Each tissue sample was then assigned a value corresponding to the ratio between the counts in the TCA-insoluble and TCA-soluble fractions, the ratio being directly

proportional to the degree of protein synthesis. All counting was done over a period of 10 min., external ratio mode, on a Hewlett-Packard scintillation counter.

II. Measurement of 5-hydroxytryptamine (5HT) uptake by rat cortex

The method used was based on that of Bjorklund et al. (304). Male Piebald Virol Glaxo strain rats, 10 weeks old and weighing from 200-230 g were operated on and given cobalt implants as previously described. Twenty days after the operation these experimental animals and 'glass-implanted' controls were stunned and decapitated. The brains were removed and immediately washed in ice-cold Krebs-Ringer bicarbonate buffer (pH 7.4) (for composition see 'Materials'). After immersion for 1 min. each brain was removed from the buffer and placed on an aluminium block chilled at 0°C on ice. The cerebellum was removed, and the cortical hemispheres separated. A Perspex microscope slide with a central trough 1 mm deep, 20 mm wide, was pressed down gently over the superior surface of each cortical hemisphere. A scalpel blade was then passed slowly with a sawing motion along the inferior surface of the slide. In this manner cortical slices of a standard 1 mm depth were obtained. From these slices circular tissue sections were cut using a thin-walled stainless steel tube with an internal diameter of 3 mm.

Each cortical disc was then placed in a numbered scintillation vial containing 2.0 ml of ice-cold Krebs-Ringer buffer solution (made up as in 'Materials'). This was pre-saturated with 95% O_2 and 5% CO_2 and had a final pH of 7.4.[1] Sections from the experimental and control animals were then pre-incubated in the buffer solution for 10 min at 37°C. In order to give a measure of 5-HT uptake attributable

to simple diffusion, other sections from control animals were incubated throughout at 0°C. To each vial was now added, using a Pederson pipette, 1 µl of a tritiated 5-HT solution (1,2-^{3}H 5-HT binoxalate, 5.7 Ci/mmol, New England Nuclear). This gave a final concentration of 5 x 10^{-8} mol/l in the incubation medium. The incubation was allowed to continue for 10 min. The reactions were then stopped by placing the scintillation vials in ice-water. Two 10 µl samples were taken from the incubation medium in each vial, for duplicate determination of the free 5-HT and metabolites. The samples were added to 10 ml Triton scintillant ('Materials') and the counts obtained hereafter referred to as the "Medium count". Each tissue section was aspirated from the scintillation vial into the barrel of a clean Pasteur pipette, and placed on a Millipore filter (Pore size 0.45 µ). The sections were then washed rapidly with 10.0ml of 0.9% NaCl under suction. After washing, each filter was removed (with the tissue section) from the Millipore filter holder, and placed in a scintillation vial containing 1.0 ml distilled water. Ten ml of Triton scintillant (made up as in 'Materials') was added to each vial, and the vial was then agitated for 15 min. in a shaker. The tissue disc disintegrated after approximately 5 min. but at this stage a slight opacity might still be detected in the scintillant solution. After fifteen min. the scintillant mixture became completely clear, indicating effective dissolution of the tissue. Counts obtained from such samples are referred to as "Tissue counts". All samples were counted for 10 min. external ratio mode, on a Hewlett-Packard scintillation counter. Each tissue section was assigned a value determined by the ratio of tissue counts to medium

counts, from which was subtracted the $0^{o}C$ control ratio, this latter factor representing the degree of passive diffusion of 5-HT into the brain slices. This gave a final tissue counts/medium counts ratio which referred only to the degree of 5-HT taken up actively by the brain slices. No monoamine oxidase inhibitor was used (as in the original method, 304). This meant that the tissue and the medium counts probably included not only ^{3}H as (^{3}H)5-HT but also (^{3}H)5-HIAA. The degree of 5-HT oxidation in these sections is not known exactly; but if 5-HIAA is more easily lost from brain sections than 5-HT, then a reduced MAO activity might cause an artificially high tissue/medium counts ratio. This potential weakness in the method is discussed in the results section.

III. Estimation of cobalt in rat brain

The estimation was carried out by flameless atomic absorptiometry (Slavin and Fernandex, 575), using a Perkin-Elmer Atomic Absorptiometer model HGA-360 with a graphite furnace attachment. Essentially the apparatus consists of a graphite tube, through the long axis of which is passed light of a wavelength that will be absorbed by the atoms of the element to be measured, the degree of absorption being monitored by the output from a photomultiplier tube. In order to measure trace elements present in biological material, a small volume of a suitable extract of the material is injected into the graphite tube. Suitable setting of the instrument allows controlled heating of the graphite tube to temperatures suitable for evaporation and then ashing of the sample, and eventual atomisation of the element of interest.

The following method was developed for the estimation of cobalt in brain tissue. Preliminary experiments were conducted to select a suitable programme for the instrument. Normal brain tissue was digested in conc. nitric acid (BDH Analar), 2 µl/mg tissue, for 2 h at 70°C in a stoppered 1.5 ml polycarbonate reaction tube. To the digest was added 18 µl/mg tissue of a standard solution of cobaltous chloride, 1 µg Co^{++}/ml. Of this mixture, 20 µl samples containing 18 ng cobalt were introduced into the instrument. Suitable conditions were found to be 90 sec. at 85°C, (for evaporation), followed by a controlled increase in temperature, over the next 90 sec. to 1,050°C, this temperature being maintained for 10 sec. (ashing stage); the temperature was then raised to 2,760°C for 10 sec. to atomise the sample. Lengthy experimentation involving adjustment of durations and temperatures of the first two stages was required to define conditions so that the on-line absorptiometer just gave nil readings until the third stage, controlling atomisation, was activated. In follow-up experiments with samples containing 50 ng cobalt 'nil' readings were obtained during the first two stages, indicating no loss from this relatively large amount of cobalt during the evaporation and ashing stages. A cathode-less light tube with a primary wavelength of 240.7 nm was used as the light source. (These lamps are supplied by Perkin-Elmer for use in conjunction with the atomic absorptiometer.)

For the estimation of cobalt in brain tissue from experimental animals, weighed pieces of brain (15-20 mg) were digested in conc. HNO_3 as described above. Portions of the clear digest were diluted 20-fold with deionised distilled water, and 20 µl portions of the dilution used for analysis. No cobalt (< 1 ng) was detected in blank digests,

with no brain tissue, nor in samples of brain from control animals, indicating no contamination from the nitric acid, deionised water or reaction tubes, and the absence of measurable endogenous cobalt. The smallest amount of cobalt that could reliably be detected in a 15 mg sample of control brain was 1.5 ng.

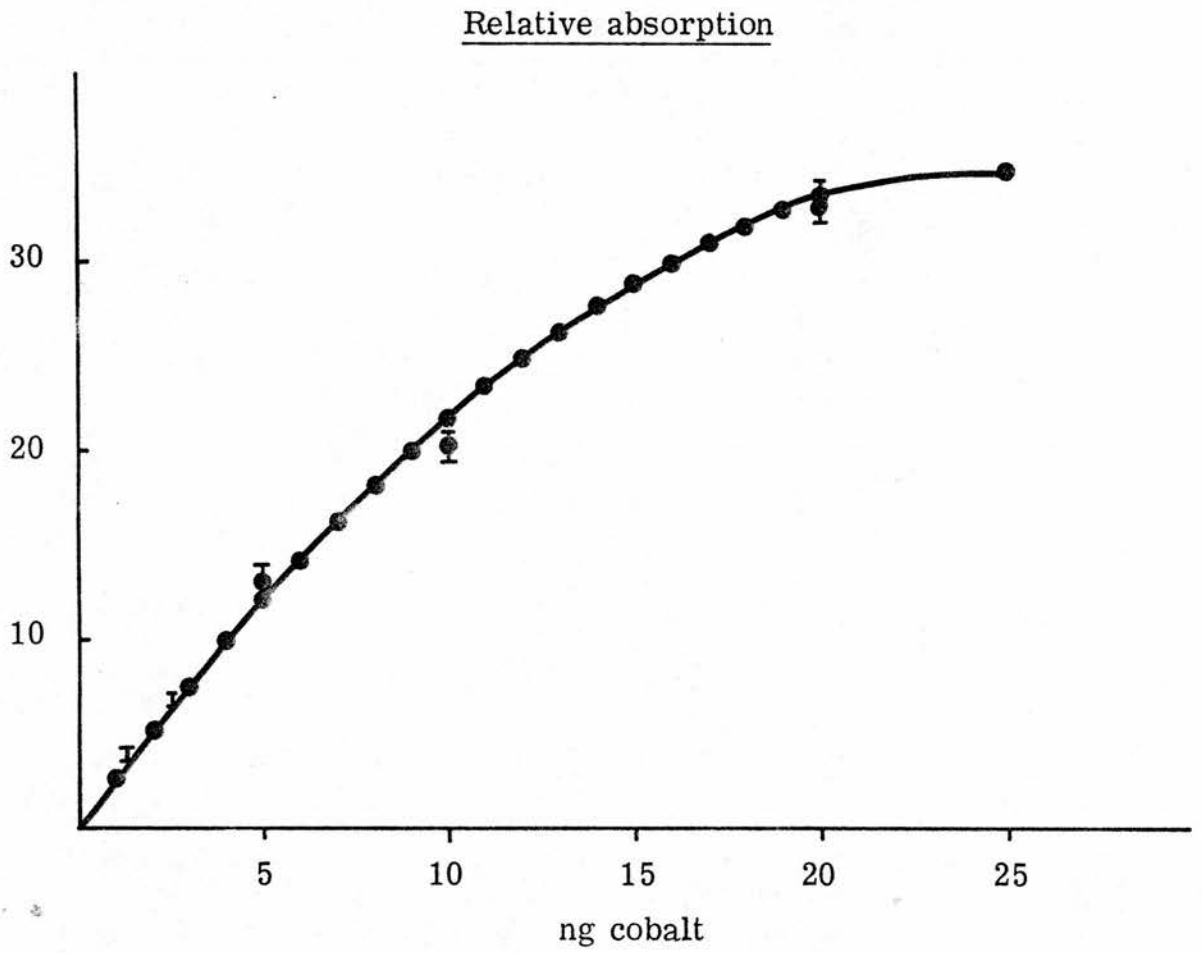
In the case of tissue samples of ipsilateral cortex from cobalt-implanted animals, the glial capsule and any calcified tissue was rejected, as were any visible traces of gelatine-cobalt.

CALIBRATION OF THE ATOMIC ABSORPTIOMETER

A series of aqueous standards of cobaltous chloride was processed through the cobalt assay method, and it became apparent that the relationship between the amount of cobalt present and the cobalt related light absorption was non-linear. Assuming a curvilinear relationship, and force-fitting the curve through $x = y = 0$, an equation was derived using the method of least squares analysis. The curve thus derived obeyed the equation $y = 2.683x - 0.052x^2$, and is shown in Figure 19, together with the experimental data. The fact that the cobalt standards of 5 and 10 ng do not fit the curve as accurately as the other standards may be explained by either postulating a dilution error committed during the preparation of the standard solutions, or by adding an x^3 and log.x factor to the equation $y = ax + bx^2$. This latter alternative however does not agree with the data published by Slavin and Fernandex (575) who found a simple $y = ax + bx^2$ curve adequate. The possibility of a consistent (in view of the results) error in the preparation of the standards, perhaps due to inaccurate calibration of one of the dispensing pipettes, cannot be discounted.

Fig. 19

Calibration of the Atomic Absorptiometer



The curve as described by the equation $y = 2.683x - 0.052x^2$ accounted for 99.3% of the variation in y.

IV. The technique of tryptophan loading (as used in the Pharmacology section)

Tryptophan was prepared for injection by the method of Ashcroft et al. (180). A known amount of L-tryptophan (200-500 mg) was placed in a mortar and ground with a few drops of Tween 80, and then 0.9% NaCl was added drop by drop with further grinding until a suspension of 150 mg L-tryptophan/ml was obtained. Each rat was then weighed and given an i.p. injection of the suspension of a volume calculated to give a dose of 400 mg/kg. A wide-bore needle was used to minimise the risk of the suspension clogging the needle.

Rats were killed 2 hours after injection, and samples of brain removed from tryptophan assay.

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION TO THE BIOCHEMISTRY OF EPILEPSY

Various studies have demonstrated disturbances of cerebral metabolism associated with idiopathic epilepsy. Amongst the metabolic changes usually referred to are reports of a reduced activity of cholinergic metabolism (390, 451, 452, 453), an inhibition of glucose metabolism, and alterations of amino acid metabolism (453, 452, 451, 390). It has not been possible to classify these changes as aetiological or associative, and there are currently no anticonvulsants in use which could be said to have been developed on the basis of these findings. More recently, interest has been focussed on the postulated central neurotransmitters, primarily gamma-aminobutyric acid (GABA) and the monoamines noradrenaline(NAd) dopamine (DA) and serotonin (5-HT).

There is a need for drugs that will interfere with the circuitry involved in the initiation and spread of epileptic behaviour, while leaving normal neuronal function undisturbed. If any one class of neurone could be shown to be specifically involved in the epileptic process, this would then serve as a pharmacological focus around which the search for specific anti-convulsants could be based. The most readily available methods of classifying distinct sub-groups of neurones, and manipulating such sub-groups, refer to the metabolism and function of the different neuro-transmitters; and there is already available a large body of information concerning the relationships between various parameters of one or other of the biochemically classifiable sub-groups and the tendency to the development of

epileptic symptoms, in a wide range of animal models and in the clinical situation. This area of research, into which the body of the work referred to in this thesis falls, is reviewed in the introductions to the relevant sections on neurotransmitter metabolism in one animal model of epilepsy, the cobalt-implanted rat.

GENERAL PHYSIOLOGY OF EPILEPSY

A large volume of research has also centred around the physiological responses associated with epileptic behaviour in various models. These appear to be widespread (occurring in and also outwith the focal areas) and complex, varying not only from model to model but also in time, different responses being associated with different stages in the initiation and development of the epileptic focus. Much of the data reported on below was assembled from the study of the consequences of clinical and experimental cranial trauma.

During the first minutes or hours after injury, humoral mechanisms may be important: Bornstein (389) demonstrated that experimental head trauma resulted in a release of acetylcholine (ACh) into the CSF; and Tower and McEachern (390) showed that in clinical cases of head injury there was also an increased efflux of ACh into the CSF. Significantly, ACh not only has a direct excitatory effect on cortical neurones (391), but has also been reported to activate human epileptic foci after topical application (392).

At later stages, for example after the recovery of consciousness, a different contributory factor has been suggested by Dempsey and Morrison (393). They suggested that injury could, by temporarily reducing the inhibitory tone of the mesencephalic reticular system

on ventro-lateral thalamic nuclei and diencephalic nuclei, release a cortical recruiting response that generally increases cortical excitability. Later again in the syndrome physiological factors related to the displacement and disorganisation of the nervous tissue may become important; venous sinus thromboses with consequent stasis and anoxia, the pressure increase following sub-dural haemorrhage, and the nervous irritation caused by the presence of sub-pial extra-vascular blood would all increase the tendency to the development of fits (394, 395). The final pathology may be contributed to by scar formation associated with alterations in local blood supply (397), and also denervation hypersensitivity phenomena (396). Finger et al. (582) suggested that the reinnervation of damaged areas of CNS by the sprouting of neighbouring, undamaged cells could introduce 'noise' and disrupt normal activity. This may also become important in later stages of an epileptic focus. The physiological responses associated with the epileptic condition precipitated by frank neuronal damage can then be described as an overlapping sequence of adjustments to the original trauma. Similarly, seizure behaviour as a consequence of cerebral trauma, and probably in other epilepsy models, may be influenced by different contributing factors at different stages after the injury. One might also hypothesize that the epileptic behaviour accompanying the early, more rapidly developing, responses to injury should be less likely to persist than those associated with the long-term resolution of the injury.

REASONS FOR CHOICE OF MODEL

The different models that have been used in the investigation of epilepsy all have their own unique advantages and disadvantages. Many types display epileptic behaviour only for a very short time, and into this category fall most of the epilepsies precipitated by systemically administered convulsants, and also the epileptic states precipitated by those substances which are effective convulsants when applied directly to the brain tissue, such as the penicillins and oestrogens. These models would not have been suitable for my purpose, as the intention was to examine neuronal changes in a chronic epileptic focus such as is found clinically. The longer term models available include the freeze lesion, audiosensitive and photosensitive epilepsies (the reflex epilepsies), and the metal lesions; in the event it was decided to investigate the metal lesion, specifically the cobalt lesion. This model is relatively cheap, and simple to reproduce, and furthermore work had already been done in the department on various aspects of the biochemistry, histochemistry and pharmacology of this model so that there already existed a framework of data against which the findings of mono aminergic involvement could be contrasted and compared. This study then, was an attempt to uncover correlations between changes in various parameters of specific types of neurone and changes in the electrical behaviour of the cortex as measured in the EEG associated with the initiation, development and eventual resolution of the cobalt epileptic focus.

It is impossible to say exactly how valid this particular model is but there is a body of information that suggests that the cobalt-implanted rat behaves in a somewhat similar way to certain cases of

human epilepsy. In an extensive study on the patterns of EEG behaviour of cobalt-treated rats, Dow et al. (456) suggested that the abnormal hypersynchronous focal epileptic activity characteristic of this model approximated very closely to the epileptic EEG patterns of some human epilepsies; this was supported by two later studies, by Dawson and Holmes (457), and Chocholova and Rodel-Weiss (458). Ashcroft et al. (354) have found that ethosuximide, an anticonvulsant used in the treatment of petit mal epilepsy, is also successful in reducing epileptic signs in the cobalt-implanted rat, while phenobarbitone and diphenylhydantoin, the two major drugs of choice in the treatment of grand mal, are not effective in this model. This pattern of response to anticonvulsant drugs plus the fact that the cobalt-implanted rats display fore-limb and whisker twitch but only very rarely develop generalised convulsions, suggests that the model may be more suitable as an analogy for petit mal or myoclonic epilepsy. The fact that one at least of the clinically used anti-convulsants is effective in this model also suggests that, even if the initial epileptogenic stimuli are different, the information concerning the development of the EEG epileptic behaviour in the rat may well be of relevance to the clinical situation. The fact that the cobalt-implanted rat only displays myoclonic behaviour may well be an advantage from the point of view of a biochemical analysis of the model, as the pathological situation and biochemistry are not compounded by the effects of generalised convulsions (466, 577).

THE COBALT MODEL - POSSIBLE MECHANISMS

The direct application of epileptogenic agents to the pial surface of the brain is a common experimental method of producing seizure discharges. The oestrogen and penicillin convulsants are applied in this way (291, 292) but these epilepsies, as already mentioned, are of too short duration to be of use in the study of the chronic epileptic focus.

Chronic focal epilepsy can be produced in the rat and in a number of other species by the application of a gelatine suspension of metallic cobalt in powder form to the cerebral cortex. Cobalt wire and cobalt metal powder alone have also been used (371, 372, 373, 374). The animals develop epileptiform EEG abnormalities associated with myoclonic whisker and limb twitches and a lowered threshold to systemic convulsants (375) within about a week after implant. These models present with substantial cell damage involving a focal area of leptomeningeal inflammation (376) and a cortical necrosis characterised by neuronal degeneration, gliosis and ingrowth of new blood vessels (377). It has been difficult, in consequence, to isolate the epileptogenic mechanism, because neuronal damage alone is often sufficient to initiate epilepsy, as is instanced by the number of patients whose epilepsy is attributable to cerebral trauma. Various authors have claimed that the effect of the cobalt ion is the critical factor (371, 378), while others suggest that the formation of the meningeal cicatrix is important in developing epileptic activity (377, 376). It seems likely that both factors, the cobalt toxicity and the general cell damage, contribute towards the epileptic phenomena, the importance of the contribution of each factor varying at different stages of the epileptic process.

However, it may well be that the crucial influence is that of the cobalt ion; glass implants, for example, although capable of inducing a degree of gliosis and meningeal damage, do not initiate epileptic foci (374), while a recent paper by Willmore et al. (379) demonstrated very clearly that the cobalt ion does possess a potent epileptogenic effect, when applied directly to cortical tissue. The epileptic signs at later stages, for example at 10 weeks post-implantation, may not be so closely linked to the presence of the cobalt ion. At this later stage atomic absorptiometry studies, reported in this thesis, show that the cobalt has been cleared from all areas of the brain except the primary focus, where some of the cobalt remains encapsulated in a calcified glial capsule. Epileptic activity at this time probably reflects the cell damage, a large degree of which may be permanent. This raises several problems concerning the mechanisms of this form of epilepsy. Is the epileptic behaviour of the first few weeks, possibly elicited by the cobalt ion, mediated by the same mechanisms as are involved in the later stages of the cobalt model when tissue scarring and/or resolution may predominate as the cause of the epileptic behaviour? If the mechanisms are different, does this mean that different stages of the development of an epileptic focus may be best treated using different drugs? It may well be that, during the early stages of the cobalt focus, metal ion chelators would be effective, or alternatively drugs acting on those groups of neurones that are most involved at this stage: but this does not mean that the chelators or the specific drugs will be effective during later stages when the epileptic mechanism may be different. Certainly the studies reported in this

FIG A1.

Spiking rates in ECoG of cobalt-implanted rats

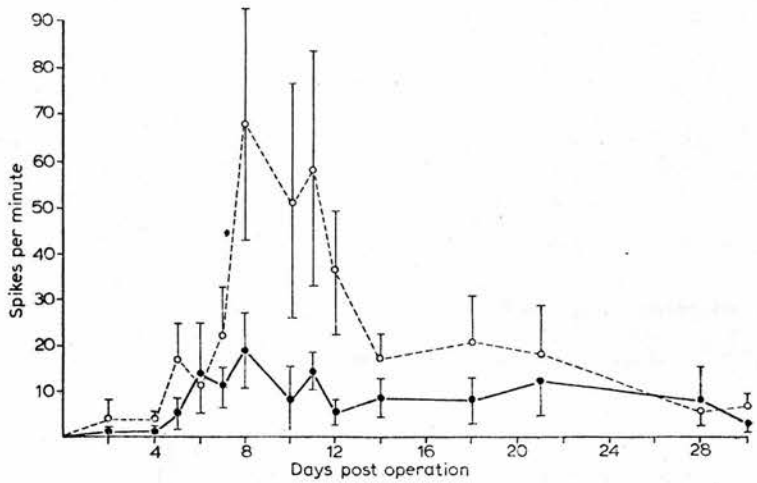


Fig. 4. Development of abnormal epileptic spike activity in cobalt implanted rats. Each point is the mean ± S.D. for the primary focus (●—●) and secondary focus (○- - - - -○). The number of animals used were for days 2, 4, 5, 6, 7, 11, 12 and 28 — 12 animals; for days 8, 14, 18 and 21 — 24 animals.

thesis do suggest that the pattern of involvement of the different neuronal types does vary at different times after the cobalt implant.

Considerable reservations must therefore be made when comparing data from the cobalt model with clinical data; much of the work done on this model has concentrated on a period of only 30 days after the implant, and it is possible that this study may shed very little light on the problem of long-term chronic human epilepsy. However, it may be of some value in unravelling the mechanism involved in the developing stages of the human epileptic condition, and in suggesting some possible avenues of research into the development of early, possibly prophylactic, treatment in cases of cerebral trauma.

TIME COURSE OF DEVELOPMENT OF EPILEPSY IN THE COBALT-IMPLANTED RAT

Following unilateral implantation of cobalt into the frontal cortex of the cat, a primary epileptic focus developed in the nervous tissue around the implant zone, and a secondary focus formed in the corresponding area of the contralateral cortex, as measured in the animals' EEG. Epileptiform waves, referred to as 'spikes' because of their typically small peak angle, appeared in both primary and secondary foci at around 3-5 days post-implant and increased in frequency until reaching a maximum in both foci at days 7-12 post-implant. The frequency of spiking in the secondary focus is almost always greater than in the primary focus. Very often the rate of spiking fell on both sides to 20-30% of peak values by days 14-16, and remained at approximately these rates for a period of 2-3 weeks, when a farther peak has been reported to develop (Fig. A1). Studies by Emson and Joseph (466) have shown that the levels of glutamic acid decarboxylase (GAD) and choline acetyltransferase (ChAC), enzymes associated with

FIG. A2 Involvement of various enzymes in cortex of cobalt-implanted rats

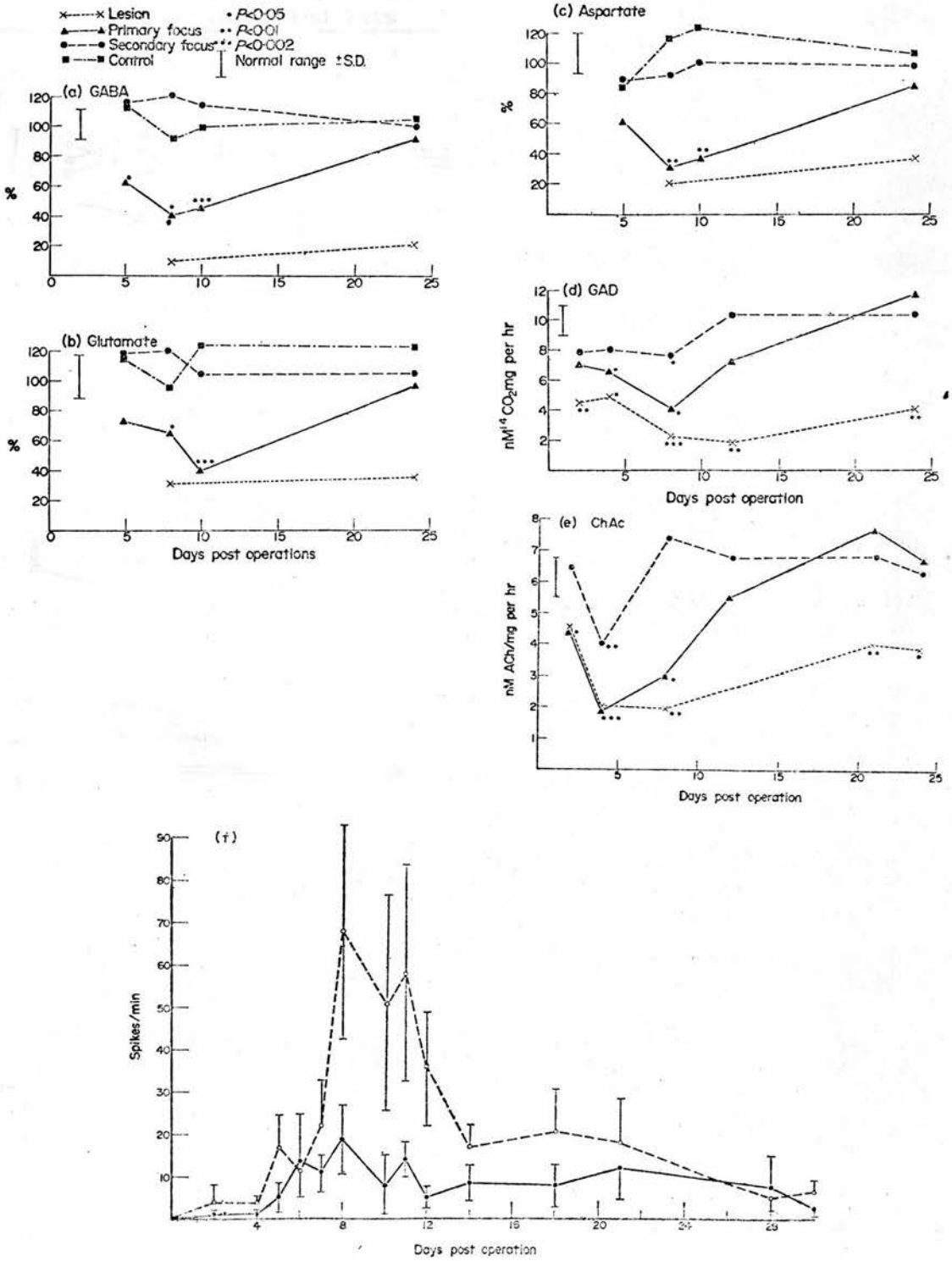


Fig. A comparison of amino acid and enzyme changes in cobalt epileptic rats relative to the development of epileptic spiking (a) γ-aminobutyric acid, (b) glutamate (c) aspartate (d) glutamic acid decarboxylase (e) choline acetyltransferase (f) spike development in the secondary focus (○- - - -○) and primary focus (●——●).

FIG. A3

Involvement of various amino acids in cortex of cobalt-implanted rats

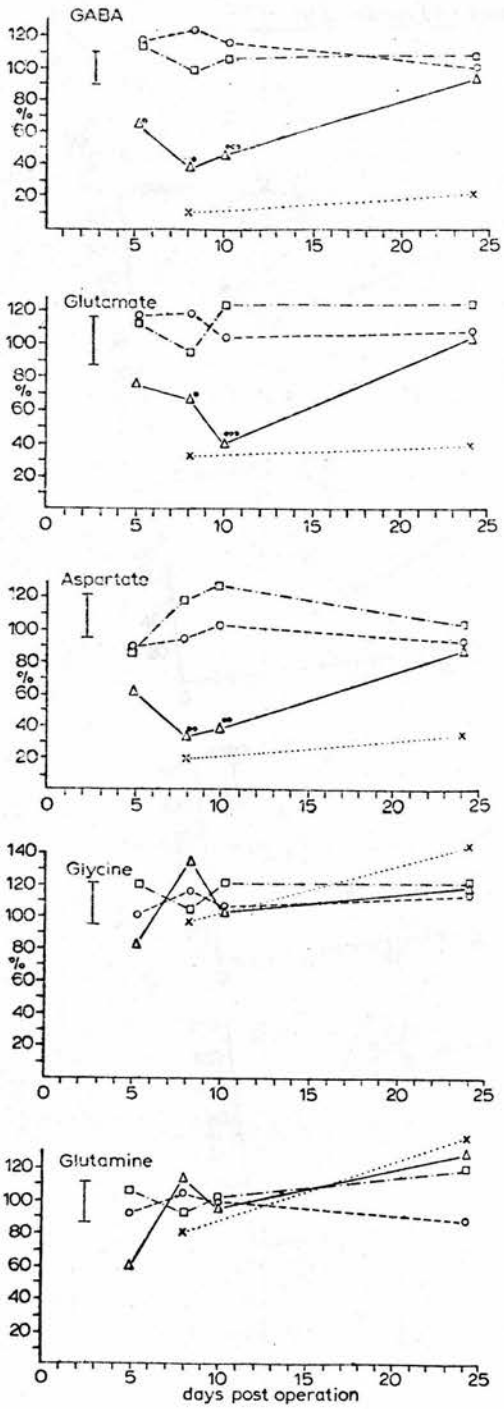


Fig. 5. The effects of cobalt implantation into the frontal cortex on amino acid levels in the lesion (×.....×), primary focus (△——△), secondary focus (○.....○) and glass implanted controls (□-·-·-□). Each point is the mean of 4 animals. Calibration bars are the normal range ± S.D. for 6 control animals. Comparison with controls, Student's *t*-test, • $P < 0.05$; •• $P < 0.01$; ••• $P < 0.002$. (466).

gabaminergic and cholinergic systems respectively (583), are reduced in the primary focal area, that the reduction is maximal when epileptic spiking is at its peak, and that the enzyme levels recover rapidly as spiking declines (Fig. A2). In parallel with these changes in enzyme activity, the concentrations of several putative transmitter amino acids including glutamate, aspartate and gamma aminobutyric acid (GABA) also fall in the primary focus area as the spikes increase, and recover as spiking decreases (Fig. A3). These changes are not consistently paralleled in the secondary focus. ChAC and GAD activity show minor reductions, as does the concentration of aspartate, while GABA levels are slightly elevated compared to controls. This may mean that the changes found in the primary focus are more a reflection of the trauma initiated by the cobalt implant than a necessary concomitant of the epileptic process. Nevertheless, Emson suggested (577) that these results might indicate that the epileptic behaviour in this model represents a denervation response. This pattern of recovery within 30 days is repeatedly referred to in later sections on monoaminergic involvement in this model, as it became apparent during the course of the work of this thesis that catecholaminergic and serotonergic parameters followed different and specific patterns of their own.

The behavioural responses after cobalt implantation are as follows; animals typically develop clonic movement of the contralateral forelimb at 4-10 days after implant, with bilateral whisker twitch. Ipsilateral forelimb twitch occasionally occurs, associated with discharges in the secondary focus (347). These traits are often still observable 3 months or more after the cobalt implantation.

THE CONTRIBUTION OF DIFFERENT TYPES OF NEURONE TO THE EPILEPTIC RESPONSE

GABAMINERGIC SYSTEMS AND THE SEIZURE THRESHOLD

The first evidence establishing γ-aminobutyric acid (GABA) as an inhibitory neurotransmitter was obtained from studies of the crayfish neuromuscular junction (Otsuka et al., 1966, 418). Later iontophoretic studies by Krjnevic and Schwartz (419) suggested that GABA might also function as an inhibitory neurotransmitter in the mammalian CNS. This idea was supported by localisation studies (412), finding that mammalian synaptosomal preparations were very rich in GABA, and by perfusion experiments (410) finding that the amount of GABA released into dog cortical perfusate or CSF was inversely proportional to the degree of cortical arousal. Histochemical work (520, 521) showed that the activity of glutamic acid decarboxylase (GAD), GABA's synthesising enzyme, correlated closely in the brain with the distribution of inhibitory neurones. Today the existence of inhibitory gabaminergic neurones in the mammalian CNS is generally agreed on, and they are thought to be involved in the epileptic condition.

One would expect that raising the effective levels of a cortical inhibitory neurotransmitter would raise the seizure threshold, and a reduction of inhibitory tone to have the opposite effect. This is in the main borne out by the experimental data. GABA injected intracranially into the frontal lobe has been shown to protect mice from

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Footnote I
Gabaminergic neurones are those neurones that employ GABA as a neurotransmitter.

electroshock and from pentylenetetrazole seizures (59). The pial application of gamma-aminohydroxybutyric acid (a GABA analogue) suppresses the activity of penicillin-induced foci in the mouse (14,15). Conversely, bicuculline, a drug thought to act as a GABA-receptor blocking agent, has been shown to act as a powerful convulsant (444, 445).

The seizure threshold in various models has also been shown to be affected by drugs that alter the synthesis or catabolism of GABA. In fact as early as 1959 Baxter et al. (415) had suspected that several of the clinically useful anticonvulsants acted by altering GABA levels; however, it is doubtful whether any anti-convulsant acts solely via the gabaminergic system, as they have been shown by other workers to exert effects on a number of other systems. This point is discussed at greater length in the section dealing with 5-hydroxytryptamine (p133).

Gamma-aminobutyric acid transaminase (GABA-T) and glutamic acid decarboxylase (GAD), respectively the catabolic and synthetising enzymes which between them control brain GABA levels, are both dependent on pyridoxal phosphate as a co-factor, as are all the transaminases, decarboxylases, and certain other enzymes. The Km of GAD, while high compared to most other known Km values for the co-factor, is lower than that of GABA-T (446, 447). This means that a decrease in the amount of available co-factor will tend to affect GABA-T first, before GAD is affected - with a consequently biphasic action on the seizure threshold. Amino-oxyacetic acid, a drug known to act as a pyridoxal phosphate antagonist, has been shown to have a moderate suppressant effect on spiking in the cobalt-implanted

rat at low doses and to be convulsant at higher doses (568). Various other pyridoxine antagonists, including 4-deoxypyridoxine and pyridoxal phosphate-glutamyl hydrazone, have been shown to act as convulsants at high doses (115, 448). Their ability to lower the seizure threshold correlated well with their ability to reduce GABA concentration in the brain. Schlessinger and Schreiber (60) also compared the rate of GABA loss with the lowering of the seizure threshold in mice on a vitamine B_6 deficient diet, and found a very close correlation between the two trends. It seemed possible that 5-HT, NAd and DA, all of whose biosyntheses include a pyridoxal phosphate-dependent enzyme, might also be involved in this lowering of the seizure threshold. However, Uchida et al., 1964 (449) found that the concentrations of the monoamine species were relatively unaffected after convulsant doses of pyridoxine antagonists, and therefore their contribution to the lowering of the seizure threshold in this model is probably unimportant.

Some workers have criticised attempts to relate seizure threshold to any factor as vague as "total brain GABA levels"; for example thiosemicarbazide, which blocks GABA synthesis, can still induce seizures in rats with artificially elevated brain GABA levels (248, 249, 250). This apparent paradox can be resolved by the suggestion that the effective inhibitory GABA is not a function of the whole brain pool, but rather a function of the synaptic, newly synthesized GABA (450), and that this may not correlate with total brain GABA. Very recently di-n-propylacetate (sodium valproate), a competitive inhibitor of succinic dehydrogenase (SDH), has been used in clinical trials in the treatment of various human epilepsies.

This drug increases cerebral concentrations of succinate, a compound formed in the transamination of GABA, and subsequently causes a reduction in the rate of the transamination of GABA in accordance with Le Chatelier's principle. The results have so far been encouraging (413, 481), in contrast to the earlier attempts at using pyridoxine antagonists as anticonvulsants, which had failed because of these compounds' lack of selectivity. Altering brain GABA levels by interfering with SDH may turn out to be amongst the more fruitful areas of anticonvulsant research, although it should be pointed out that some doubt exists concerning the mode of action of di-n-propyl-acetate. Therapeutically effective doses in man are of the order of 5-10% (in terms of mg/kg body weight) of the dose levels found to be effective in raising rat brain GABA (Emson, P., private communication). This is by no means conclusive evidence, however, as very little work has been done on the possibly considerable species differences that may exist in terms of the absorption, metabolism, distribution and clearance of this drug.

SEIZURE THRESHOLD AND CENTRAL CATECHOLAMINERGIC NEURONAL SYSTEMS

Many workers have suggested that the seizure threshold in a wide range of animal models of epilepsy is proportionally related to, amongst other factors, central levels of the postulated catecholamine transmitters.

The evidence has been mostly derived from studies of drugs, known to act on central catecholamine systems, that have been found to alter the seizure threshold. A second important category of data derives from studies of various parameters of amine metabolism in a range of models of epilepsy, including clinical cases. A third category

includes the work that has attempted to unravel the mechanism of action of the various convulsant and anticonvulsant drugs, documenting effects on the catecholamine systems amongst others; this work is in general less clear-cut than is the information provided by the first two categories, because the anticonvulsants in particular have been demonstrated to have a wide spectrum of effects only some of which involve the amine systems.

DRUGS AFFECTING CATECHOLAMINERGIC NEURONAL FUNCTION

a) Drugs that reduce catecholamine levels.

Reserpine and tetrabenazine have both been used to examine the effects of central amine depletion on the seizure threshold. Both drugs reduce central concentrations of 5-HT as well as of NAd and DA, and both drugs lower the seizure threshold in a variety of models, including pentylenetetrazole convulsions (38, 89, 90, 91), electroshock (99), audiogenic shock (95, 96, 97, 98) and convulsions induced by exposure to hyperbaric oxygen (92, 93).

Somewhat more specific amine depletors have also been examined; the fall in NAd synthesis after dopamine β-hydroxylase inhibition has also been reported to lower the seizure threshold in the audiogenic mouse model (44, 145, 146) and the actual destruction of central catecholaminergic neurones using 6-hydroxydopamine, a fairly selective catecholamineraic neurotoxin, has a similar but longer-lasting effect (144). A reduction of monoaminergic tone can also be achieved by the use of specific receptor blockers. The antipsychotic phenothiazines have been shown to exert a wide spectrum of effects, including a central DA- and NAd-receptor block and this range of drugs has been found not only to lower the seizure threshold

in animal models (109) but actually to precipitate fits in certain clinical cases of epilepsy (110, 147).

b) Drugs that raise catecholamine levels.

L-Dopa loading has been shown to raise the seizure threshold in a number of models of epilepsy. It was found to raise the threshold to audioshock in reserpinised, audiosensitive mice (120, 121), and intra-ventricular injections of L-Dopa have been used to raise the seizure threshold to pentylenetetrazole in reserpinised mice (54). However this last study reported a biphasic action of L-Dopa, namely a protective action at high doses but a facilitating effect at low doses. Schlessinger et al. (59), using the mouse audioshock and pentylenetetrazole models, demonstrated a seizure threshold-raising effect of NAd, given intra-cranially into the frontal lobe. Monoamine oxidase (MAO) inhibiters have also been used to raise central amine levels; although the evidence here is not so clear, as not only NAd and DA but also 5HT, and in fact levels of all biogenic amines normally metabolised by MAO, are altered. However, various workers (100, 101, 102) have suggested that the anticonvulsant effects of MAO are mediated at least in part by the increase in central levels of NAd and DA.

Another category of data concerns the use of various drugs thought to increase effective, if not absolute, amine levels. It has been suggested, for example, that the mode of action of the tricyclic antidepressants is via an "amplification" of central catecholaminergic neurones brought about by blocking amine re-uptake at nerve terminals. It is of interest in the present context that imipramine has been reported to be effective in the treatment of some cases of petit mal and minor motor epilepsy (100).

Various studies have also looked at the effects of monoaminergic receptor stimulators. Apomorphine, ergocornine and piribedil, for example, have all been shown to raise the seizure threshold in audio-sensitive mice (121), and in related work it has been shown that amphetamine, which stimulates central NAd and DA release, can protect audio-sensitive mice from audio-shock (123), and can also reduce spiking in the EEG of the cobalt-implanted rat (125).

The data so far summarised support the original hypothesis that the seizure threshold is linked to central catecholaminergic activity. This is also supported by microiontophoretic studies, (75, 76, 77, 78), all of which indicate that NAd and DA act as inhibitory neurotransmitters in the cortex. It must be admitted, however, even those drugs which are thought to have the most specific mode of action may well exert effects on more than one system. Some of the treatments may act directly on 5HT systems - for example reserpine and tetrabenazine. Others may affect the 5HT systems indirectly. It has been established that functional links exist between noradrenergic and serotonergic systems in certain animals; in the cat, sectioning the noradrenergic dorsal bundle apparently results in an increased cortical and raphe synthesis of 5HT (380, 511).

There is also reason to believe that drugs acting on NAd and DA neuronal systems in the brain have an effect on intra-cerebral blood flow, and hence may alter regional metabolism not specifically related to catecholamine systems; this is discussed in more detail in the section on 5HT (Ref.437). It should also be pointed out that L-Dopa loading techniques raise central levels of both NAd and DA, but have also been reported to cause a reduction in whole brain 5HT

in the rat (589). Many MAO inhibitors and monoamine uptake blockers are too unspecific to permit differentiation of the effects of the monoamine neurotransmitters.

There are available some studies that have attempted, in view of the above criticisms, to provide more specific answers. Navarro et al., (336) have shown that phentolamine and sotalol, α- and β-adrenoceptor blockers respectively, have an anti-convulsant effect in the mouse electroshock model despite their action mimicing a reduced noradrenergic tone. Inversely, although MAO inhibitors and monoamine neuronal uptake blockers raise monoamine levels absolutely or effectively, both have been shown to precipitate, on occasion, fits in epileptic patients (102, 103). Kellog et al. (139) using the techniques of catecholamine synthesis inhibition and catecholamine precursor administration, have found that the seizure threshold in the audiosensitive mouse model can be raised by increasing or decreasing central catecholamine concentrations. Some very interesting work has also been done on the cobalt rat model. Ashcroft et al. (354) demonstrated that, while DA-releasing agent amphetamine and DA-receptor stimulator apomorphine reduced or abolished epileptiform signs in the EEG, DA receptor blocker chlorpromazine and the NAd receptor stimulator clonidine had the opposite effect.

Another very clear study by Azzaro et al. (44) examined the effects of manipulating levels of specific amines on the electroshock seizure threshold in the mouse. They found that inhibiting either tyrosine hydroxylase or tryptophan hydroxylase could delay the recovery of the threshold to control values after reserpine, whereas a simultaneous inhibition of both hydroxylases could prevent

the recovery indefinitely. However, in the unreserpinised mouse, the threshold could not be affected by either synthesis inhibitor acting on its own, but only by a combination of the two. Similar results have been published by Killiam and Frey (335).

Kellog et al. (139) also found that, in a certain type of audio-sensitive mouse, audiogenic seizures could be blocked using clonidine, an α-adrenergic receptor agonist, but not by apomorphine, a DA-receptor stimulator. These findings contrast with the opposite pattern of results found in the cobalt-implanted rat, in which clonidine aggravated spiking while apomorphine reduced it (354). This may be related to the fact that morphine has a stimulatory effect in mice but a sedative effect in rats (597). This suggests that the neuronal substrate to epileptic behaviour is model-specific, or species dependent, and that the involvement of monoaminergic sub-systems (and presumably also gabaminergic, cholinergic and indeed all neuronal sub-groups) may differ widely from model to model.

Iturrian and Fink (345) proposed a model of the convulsion consisting of three separate but interdependent systems. Firstly, an afferent link, requiring the operation of an oscillator within which a substantial number of neurones must discharge over a limited period of time. Secondly, a mediating link consisting of the mechanisms responsible for the propagation of the seizure discharge from the oscillator (this mediating link has been the target of several neurosurgical techniques used clinically, and this is discussed briefly in the summary of the thesis), and thirdly, an efferent link, at which level mechanisms operate to produce a generalised seizure. Although the last stage may be common to all forms of tonic-clinic epilepsies (346), it seems logical to suppose that the neuronal substrate for the first two stages is model specific, and is not the same in, for example, electrical, chemical and reflex epilepsies.

Not many workers would now agree with the earlier, unifying hypothesis that sought to establish a common biochemical denominator whether serotonergic (330, 331, 332, 333) or catecholaminergic (328, 329, 543) to all types of epilepsy. The concept of model-specific patterns of neuronal involvement helps to explain and reconcile much of the previously irreconcilable data.

Rudzik and Johnson (55) performed an elegant series of experiments which also suggested that the various monoamine neuro-transmitters played different roles in the different epilepsy models, and pointed out very effectively the weakness of any general statement linking seizure threshold with central monoamine levels. They examined the effects of specific monoamine depletion in two different models, the mouse electroshock model and the mouse pentylenetetrazole model, and it became apparent that the two models involved two different monoaminergic systems. The pentylenetetrazole threshold could be elevated by raising effective 5-HT levels and lowered by reducing central 5-HT, but it was insensitive to manipulation of central NAd. Conversely the electroshock threshold was shown to be directly related to central NAd levels but to be unaffected by altering serotonergic tone. Similarly, in the rat, apomorphine has been shown to afford protection against maximal electroshock seizures (569) and to reduce epileptic activity in the cobalt-implanted rat (354), but it is also reported to potentiate pentylenetetrazole seizures (570).

The circuitry of even one type of convulsive model may be species dependent. Amphetamine has been reported to raise the threshold to electroshock in rabbits (543) and mice (544), while lowering it in the rat (545).

To summarize, it must be admitted that some of the data referred to in this section do not match very well. There are three main reasons for this; first, some of the earlier work reported on total brain monoamine levels, rather than on neurotransmitter turnover; second, some of the drug treatments may not have been as specific as they were thought to be, and third, the variety of models used makes it difficult to compare data from different laboratories.

FINDINGS OF ALTERED AMINE METABOLISM IN EPILEPSY MODELS

One of the first studies by Schlessinger *et al.* (356) on the neurochemistry of audiogenic reflex epilepsy described how the seizure threshold in genetically susceptible mice varied with age, and how this correlated with age-dependent changes in brain amine levels. They discovered that at the time of maximal vulnerability, brain NAd and 5HT levels were respectively 40 per cent and 50 per cent of values in non-sensitive mice while at later times when the mice were no longer audio-sensitive their brain amine levels closely matched values in other mouse strains.

There is a suggestion of similar NAd involvement in a related mouse model, the rubidium chloride-treated audio-sensitive mouse (468). Paradoxically this drug has been reported to cause an increase in NAd turnover in rat brain (469). In the cobalt implanted rat, however, studies by Colosanti and Craig (454) have failed to reveal any changes either in whole brain NAd, DA and 5HT, or in NAd and DA turnover.

ALTERED CATECHOLAMINE METABOLISM IN CLINICAL EPILEPSY

In general, central catecholamine metabolism in human subjects can only be followed indirectly. Usually only CSF samples are

available, and workers in this field have for the most part been interested in measuring levels of the dopamine metabolite homovanillic acid (HVA). The problems of interpreting the data from CSF-sampling are discussed in more detail in the section on 5HT (p130).

Shaywitz (87) and others (32, 33, 34) found a 50% reduction of HVA in the CSF of epileptic children, and a similar reduction was also found in the CSF of a groupf of unmedicated epileptic patients (9), suggesting that the low CSF HVA concentrations genuinely reflected the disease process, rather than the effects of the patients' medication.

However, there are conflicting reports; Garelis and Sourkes (8) report normal CSF HVA levels in lumbar and mixed samples taken from grand mal and temporal lobe epileptics; and Chadwick et al. (29) found CSF levels of HVA in unmedicated patients fairly close to control values. They also reported a correlation between CSF HVA levels and plasma anti-convulsant concentrations, and suggested that various anti-convulsants raised CSF HVA levels - in fact that the increase in HVA levels was a direct manifestation of the anti-convulsants' effectiveness.

THE EFFECTS OF ANTI-CONVULSANTS ON CATECHOLAMINE METABOLISM IN BRAIN

Phenobarbitone has been shown to reduce DA and NAd turnover in rat brain (61, 62, 63). This action of the drug is hard to square with the conclusions of Chadwick et al. (29), (see above), but apparently phenobarbitone (and diphenylhydantoin) also interfere with the transport of organic acid metabolites from brain which would tend to increase CSF HVA levels (36). In the mouse electroshock and pentylenetetrazole models, NAd and DA depleting agent α-methyltyrosine

and NAd receptor blocking agent phentolamine were found to render the anticonvulsants diphenylhydantoin and phenobarbitone ineffective, while L-Dopa had an opposite, synergistic effect (41). This suggested that these anticonvulsants acted at least in part via the central catecholaminergic systems. However, in conflicting experiments (140, 141) the amine depletors, reserpine, tetrabenazine, α-methyldopa, α-methyltyrosine and adrenergic blocking agents tolazoline, nethalide and chlorpromazine, had little effect on the anticonvulsant action of diphenylhydantoin in the mouse electroshock model. The authors suggested therefore that diphenylhydantoin did not exert its anticonvulsant effect solely by way of central monoamine systems. This discrepancy may be explicable in terms of the specificity of the monoamine depleting agents used, or degree of reduction of amines.

Amphetamines have also been used clinically in the treatment of epilepsy;including petit mal seizures (542); they counter the ataxia and drowsiness produced by phenobarbitone and diphenylhydantoin, and have been reported to enhance the anti-convulsant action of phenobarbitone clinically, (541), and the anticonvulsant action of phenobarbitone and diphenylhydantoin in the mouse electroshock and pentylenetetrazole models (55). The authors inferred that the action of amphetamines in these models was in some way related to the central NAd releasing effect of this class of drugs. They suggested that this was probably not the only mode of action, as D-amphetamine was capable of reversing the lowering effect of reserpine on the seizure threshold to both pentylene tetrazole and electroshock, in other words was still an anticonvulsant when central NAd stores were depleted.

It is worthwhile mentioning here that there exist theories of the action of anti-convulsants which are completely divorced from

any concept based on the biochemical sub-classification of neurones, but concentrate instead on the electrical properties of the neuronal membrane. Swinyard (357) suggested that anticonvulsants might act by stabilising the neuronal membrane, resulting in a reduced tendency to hypersynchrony. This would disrupt the oscillator mechanism and also result in a reduction of spread of the epileptic discharge, which could be thought of as dampening down the mediating and efferent links of Iturrian and Fink's model (345 (p72).

The disruption of the oscillator link, and the damping of the mediating and efferent links could, therefore, be a reflection of an alteration in the properties of the conducting nervous membrane, rather than a result of interference with events localised to the synapse. It might consequently be wrong to overemphasise the biochemical findings referred to above.

INTRODUCTION TO THE BIOCHEMISTRY OF GABA IN THE COBALT-IMPLANTED RAT

Work already carried out in the M.R.C. Brain Metabolism Unit (466) using the Finkheimer technique to examine the development of epileptic foci in the rat after the implantation of metallic cobalt into the frontal cortex, has shown that degenerating terminals, axons and cells could all be clearly demonstrated to be present in the primary lesion between the 4th and 29th day post implant; this was most marked in the involved areas of sensori-motor cortex, but cellular damage was also evident in the body of the caudate nucleus inferior to the site of implant. There was also evidence of a lesser degree of damage in the secondary focus and in the contralateral caudate nucleus. During the same period spikes were present in the ECoG, appearing 3 to 4 days post-implant and increasing in frequency until reaching peak rates between 7 and 12 days, and then falling off to 20 to 30%

of the peak rates during the third week. It was also shwon (466) that glutamic acid decarboxylase (GAD) activity was reduced in the area of the primary lesion between 4 and 8 days post-implant, while levels of GABA in the same area were reduced at 5 days post-implant, and were lowest at days 8 to 10, before recovering at day 25. Involvement of both of these parameters in the secondary focus was less conclusive. The degree of involvement of cholinergic neurones as measured by assaying choline acetyltransferase (ChAc) activity, was also mentioned, and this enzyme also showed reduced activity in the primary focus between days 2 and 10 with a smaller, transient fall at day 5 in the secondary focus. Lactic acid dehydrogenase (LDH) activity was only reduced in the immediate lesion area, suggesting that outside the area of necrosis, nerve terminals were more specifically affected (Figs A1-A3). There appeared to be some correlation between the reduction in amino acid concentrations and enzyme activity and the developing epileptic signs in the ECoG, over the first two weeks after the cobalt implant. The altered gabaminergic parameters suggested that a permissive relationship might exist between the viability of the gabaminergic system and the tendency of the cortex to produce spike discharges. However, it was impossible to say from this evidence alone which was cause and which effect, or whether both factors were a consequence of some other change(s) occurring in the damaged tissue.

In the hope of throwing some further light on this problem it was decided to measure gamma aminobutyric acid transferase (GABA-T) activity in primary and secondary foci over the 30-day period already examined. GABA-T estimations were carried out using the method of

Table A1b

Estimation of brain GABA-transaminase activity (n.mol. GABA transaminated/g protein/ hr) in rat frontal cortex at intervals after cobalt-implantation

A) Control values (Unoperated littermates)	365 378 326 360 357 ± 19(4)	
B) Cobalt-implanted rats	Lesion	Contralateral
Day 3 post-implant	126 7 57 nil nil 76 44 ± 47(6)	288 96 138 298 392 281 249 ± 101(6)P1
Day 6 post-implant	71 nil 6 6 41 25 ± 27(5)	nil nil nil nil 26 5 ± 10(5)
Day 9 post-implant	269 886 508 721 625 602 ± 207(5)P1	63 264 264 172 119 176 ± 79(5)P3
Day 12 post-implant	239 286 435 152 553 333 ± 143(5)	126 138 50 125 nil 88 ± 54(5)P3
Day 30 post-implant	237 185 229 344 249 ± 58(4)P2	511 534 435 504 496 ± 37(4)P2
Day 100 post-implant Lesion and contralateral sides pooled	384 315 357 304 343 ± 33(4)	

Notes:

1. All final values are given as the mean ± standard deviation.
2. P-values refer to comparisons between experimental and control data, made using Student's t-test with Bessel's correction.
   P1 = $0.1 > P > 0.05$; P2 = $0.025 > P > 0.02$; P3 = $0.01 > P > 0.005$

Hall and Kravitz (574, Methods B1) in frontal cortex at different times after cobalt implantation (Fig. A4b). GABA-T activity fell in both foci at days 3-6 post-implant; then increased to above normal values in ipsilateral cortex at day 9 before stabilising at day 12. The pattern of changing GABA-T activity in the secondary focus was slightly different, remaining low until day 12, before increasing to above normal values at day 30. The published data on GAD activity and GABA concentrations (466) show no corresponding increases at days 9 and 30 in primary and secondary focus, and it was therefore considered unlikely that the GABA-T data might represent any increase in the number or activity of gaba-containing nerve terminals. It seemed more likely to be associated with the glial reaction that develops around this time in the area of the cobalt implant (466). This would provide an adequate explanation for the increased GABA-T activity, as it has been shown that a large proportion of GABA-T in brain is, unlike GAD, associated with glia (597). The much smaller increase in GABA-T activity in the secondary focus at day 9 may reflect the much smaller glial reaction on the contralateral side (466).

INTRODUCTION TO THE BIOCHEMISTRY OF THE CATECHOLAMINERGIC SYSTEMS IN THE COBALT-IMPLANTED RAT

None of the results of the previous work had been directed to examining the role of monoaminergic neurones in this model. It was therefore decided to assay enzymes involved in the functioning of catecholaminergic neurones in the primary and secondary focus areas at different times after cobalt implantation.

Tyrosine hydroxylase (TOH) is thought to be the most specific

marker enzyme, being located in the CNS only within catecholaminergic neurones (604). Monoamine oxidase (MAO) a mitochondrial enzyme (403), and catechol-O-methyltransferase (COMT) a cytoplasmic enzyme (404), were the other two enzymes studied, but although both of these enzymes play an integral role in the function of central monoaminergic systems the data they provided was thought to be less unequivocal, as they are associated not only with monoaminergic neurones but also non-aminergic neurones (401) and with glia (402). MAO in particular is rather evenly distributed in brain, with only a three-fold difference in activity reported between areas of highest and lowest activity, the hypothalamus and corpus callosum respectively (149). A recent paper by Ulmer et al., 1975 (67) casts further doubt on the usefulness of MAO as a monoaminergic cell marker; they found that in undercut cortex preparations, i.e. in tissue presumably cut off from extra-cortical innervation, MAO fell by only 10 per cent, in marked contrast to the reduction, by almost 50 per cent, of acetylcholinesterase.

The reason for assaying MAO and COMT was therefore as support for the TOH data, which on its own would have been open to various criticisms, discussed in the following section, concerning the method of assay used. To provide farther information on the involvement of catecholaminergic neurones in the epileptic process, concentrations of noradrenaline (NAd) were measured in the cortex, and metabolites of dopamine (DA) homovanillic acid (HVA) and of NAd, 4-hydroxy-3-methoxyphenylglycol (HMPG) were monitored in cortex and striatum. ToH activity was also assayed in sections of mid-brain containing the locus coeruleus to provide some indication of events at the cell body level of the noradrenergic neurones projecting to the cortex.

Table A1

Protein content (mg/g wet weight of tissue) in lesion (l) and contralateral (c) frontal cortex of the cobalt-implanted rat.

Days post-implant

Day 0	Day 3		6		9		12		30	
	l	c	l	c	l	c	l	c	l	c
113	98	114	94	99	92	93	112	106	107	120
108	103	108	97	103	101	101	93	102	113	109
121	94	110	92	103	95	103	92	113	115	112
112	97	102	107	94	93	99	102	95	110	117
106	106	117	98	97	109	114	96	92	103	112
104	101	111	105	102	97	107	96	103	116	107

Mean values (+ standard deviation)

Unoperated animals (Day 0): 110.66 ±5.58

Cobalt-implant animals

	Day 3	6	9	12	30
Lesioned cortex	99.83±3.97	98.83±5.45	97.83±6.25	98.5±6.82	110.66±4.57
Contralateral cortex	110.33±4.71	99.66±3.34	102.83±6.54	101.83±6.91	112.83±4.45

NAd concentrations were assayed using the fluoroassay of Schlumpf et al. (24), Methods CI. Metabolites HVA and HMPG were assayed using the methods of Roos (21), Methods CII, and Walter and Eccleston (562), Methods CIII respectively. The radiometric tyrosine hydroxylase assay was based on the method of Hendry and Iverson (353), Methods BIII.

INVESTIGATION OF THE CATECHOLAMINE SYSTEM IN THE COBALT MODEL

A. ENZYMES

All the enzymes referred to here were assayed in terms of enzyme activity/g protein, rather than per g wet weight, to avoid the risk that oedema developing at any stage might confuse the picture with an increase in the fluid content of the tissues sampled. The protein content of every sample was assayed using the method of Waddell (572) (Methods DIa). The results show (Table A1) that a reduced protein content/g brain develops around the primary lesion by day 3 post-operatively, and by day 6 in the secondary focal area. In both cases the protein/wet weight ratio has returned to normal values by day 30. The rapidity of the changes suggest that the altering protein/wet weight ratio is more influenced by changes in fluid content rather than by any actual changes in protein levels.

Levels of activity of all three enzymes, ToH, MAO and COMT, fell in both ipsi- and contra-lateral cortex as early as 3 days post-implant. (Tables A2,3,4). Levels of all three enzymes were still low in both cortices at 30 days, and had returned to normal values at 100 days. These results differ in two important ways from the published data (466) already referred to, concerning the cholinergic biochemistry and gabaminergic biochemistry of this model. First, MAO and TOH

Table A2

Estimation of tyrosine hydroxylase (in n.mol Dopa produced/g prot./h)

in ipsi- and contralateral frontal cortex of cobalt-implanted rats

A) Control values (Unoperated littermates)	5.0 3.6 5.0 5.3 3.8 2.2 4.2 ± 1.1(6)	
B) Cobalt-implanted rats	Lesion	Contralateral
Day 3 post-implant	0.54 0.68 nil 0.34 0.77 0.42 ± 0.27(5)	0.69 1.86 1.37 2.08 1.35 2.07 1.57 ± 0.49(6)
Day 6 post-implant	0.43 0.76 0.84 0.58 0.14 0.55 ± 0.25(5)	0.78 0.77 0.64 0.6 0.14 0.58 ± 0.24(5)
Day 9 post-implant	0.12 0.13 0.24 0.38 0.48 0.27 ± 0.14(5)	0.43 0.86 0.44 0.65 0.69 0.61 ± 0.16(5)
Day 12 post-implant	0.4 0.17 0.15 0.15 0.33 0.24 ± 0.1(5)	0.71 0.51 0.55 0.39 1.5 0.73 ± 0.4(5)
Day 30 post-implant	1.07 1.4 1.09 1.36 1.23 ± 0.15(4)	1.23 1.36 1.01 1.85 1.36 ± 0.31(4)
Day 100 post-implant Lesion and contralateral sides pooled	3.42 2.06 4.71 4.39 3.65 ± 0.89(4)	

Notes:

1. All final values are given as the mean ± standard deviation.

Table A3

Estimation of catechol-O-methyltransferase activity (n.mol. 4-hydroxy-3-Methoxyphenylglycol formed/g protein/h) in ipsi- and contralateral frontal cortex of cobalt-implanted rats

A) Control values (Unoperated littermates)	370 436 426 416 357 440 407.5±32.3(6)	
B) Cobalt-implanted rats	Lesion	Contralateral
Day 3 post-implant	92 64 49 38 36 31 52 ± 21(6)P3	72 60 54 107 48 37 63 ± 22(6)P3
Day 6 post-implant	878 898 949 317 1742 957 ± 455(5)P1	133 100 154 50 253 138 ± 67(5)P2
Day 9 post-implant	151 582 389 144 132 280 ± 179(5)	97 150 94 87 52 96 ± 31(5)P3
Day 12 post-implant	97 68 230 281 625 260 ± 199(5)	106 120 179 233 160 ± 51(4)P2
Day 30 post-implant	137 95 126 222 145 ± 47 (4)P2	418 85 165 213 220 ±123(4)
Day 100 post-implant Lesion and contralateral pooled	448 397 455 401 425 + 26(4)	

Notes:

1. All final values are given as the mean ± standard deviation.
2. P-values all refer to comparisons between experimental and control data. Student's t-test was used, with Bessel's correction.
   P1 = $0.05 > P > 0.025$; P2 = $0.02 > P > 0.01$; P3 = $P < 0.001$

Table A4

Estimation of monoamine oxidase activity (μ.mol. tyramine oxidised/g protein/h) in ipsi- and contralateral frontal cortex of cobalt-implanted rats

	Lesion	Contralateral
A) Control values (Unoperated littermates)	36 43 37 31 35 26 34.7±5.3(6)	
B) Cobalt-implanted rats		
Day 3 post-implant	3.7 3.7 7.4 5.3 4.5 5.2 5.0±1.3(6)	3.4 6.4 5.7 8.4 5.4 4.3 5.6±1.6(6)
Day 6 post-implant	7.4 3.5 18.2 3.9 13.7 9.3±5.7(5)	4.9 6.2 3.4 4.1 9.2 5.6±2.1(5)
Day 9 post-implant	1.8 6.0 4.4 1.4 2.9 3.3±1.7(5)	10.3 3.9 1.5 1.9 1.7 3.9±3.3(5)
Day 12 post-implant	2.4 1.5 0.6 0.7 nil 1.0±0.8(5)	1.8 2.1 0.2 4.7 nil 1.8±1.7(5)
Day 30 post-implant	7.4 6.4 12.0 6.9 8.2±2.2(4)	5.4 8.9 7.8 7.7 7.5±1.3(4)
Day 100 post-implant Lesion and contralateral pooled	25 24 31 38 29.5±5.6(4)	

Notes:

1. All final values are given as the mean ± standard deviation.

activities did not return to normal by 20 to 30 days, remaining at roughly 30% of normal values. Second, it appeared that these enzyme activities were as much affected in the secondary focus as in the primary focus, in contrast with the findings of relatively minor alterations in the activity of cholinergic and gabaminergic enzymes in the secondary focus. COMT activity also fell in both foci at day 3, but in this case, although levels remained low in the secondary focus throughout the first 30 days. There was an increase to over double normal levels in the primary focus at day 6, remaining substantially higher than secondary focus values at day 9 and 12. However, by day 30, they were low again and close to secondary focus values. It was thought that the increase in the primary focus might be related to the gliosis that develops around the cobalt implant at this stage (466), as COMT is associated with glia (402). This does not quite fit with the findings of increases in GABA transaminase activity in the primary focus at day 9, which was also tentatively ascribed to a glial reaction, because at day 9 COMT activity has already fallen from the peak values reached at day 6. This could be explained theoretically in terms of a sequence of biochemical events occurring in the developing population of glia as the damaged tissue reacts to the trauma of the implant. Another possibility is that there may be two distinct populations of glia involved in the response; there is some evidence for a biphasic glial response after injury, as described by Sumner and Sutherland (364), developing in the hypoglossal nucleus after hypoglossal axotomy. In this case, however, the transition occurred rather more slowly, the microglial response peaking between 2 and 14

Table A5

Estimation of 4-Hydroxy-3-Methoxyphenylglycol (HMPG) in ipsi- and contralateral frontal cortex of cobalt-implanted rate (ng HMPG/g wet weight brain)

A) Control values (Unoperated littermates)	116 136 193 148 ± 33(3)	
B) Cobalt-implanted rats	Lesion	Contralateral
Day 4 post-implant	63 165 111 68 102 ± 41(4)	84 126 78 96 ± 21(3)P1
Day 8 post-implant	801 483 449 578 ± 159(3)P3	973 877 270 707 ± 311(3)P2
Day 15 post-implant	105 90 71 39 ± 14	351 137 138 209 ± 101(3)
Day 20 post-implant	115 90 128 111 ± 16(3)	170 165 135 157 ± 16(3)
Day 100 post-implant (Lesion and contralateral sides pooled)	105 113 136 158 128 ± 21 (4)	

Notes:

1. All final values are given as the mean ± standard deviation.
2. Tissue from 3-4 rats was pooled for each analysis.
3. P-values all refer to comparisons between experimental and control groups. Student's t-test was used, with Bessel's correction. P1 = 0.2 > P > 0.1; P2 = 0.1 > P > 0.05; P3 = 0.02 > P > 0.01.

Table A6

Estimation of 4-Hydroxy-3-Methoxyphenylglycol (HMPG) in caudate nuclei, ipsi- and contralateral to cobalt implant in rat frontal cortex

(ng HMPG/g wet weight brain)

A) Control values (Unoperated) (Both caudate nuclei pooled)	136 152 193 160±24(3)	
B) Cobalt-implanted rats	Lesion	Contralateral
Day 4 post-implant	109 180 153 298 185±70(4)	137 223 278 213±58(3)
Day 8 post-implant	1098 624 499 740±258(3)P2	540 323 455 439±89(3)P3
Day 15 post-implant	231 142 185 187±36(3)	236 88 107 144±66(3)
Day 20 post-implant	114 98 135 116 ± 15(3)P1	190 165 201 185±15(3)
Day 100 post-implant	105 110 136 118±14(3)P1	113 84 158 118±30(3)

Notes:

1. All final values are given as the mean ± standard deviation.
2. Tissue from 4-5 rats was pooled for each analysis.
3. P-values all refer to comparisons between control and experimental data. Student's t-test was used, with Bessel's correction.
   P1 = $0.1 > P > 0.05$; P2 = $0.025 > P > 0.02$; P3 = $0.01 > P > 0.005$

days after axotomy and the astrocyte response between 14 and 35 days. One possible reason for the apparent compression of the time-scale in the cobalt rat gliosis may be a geometrical one - cortical trauma can stimulate a glial response in a zone of 360° around the injury, whereas in the hypoglossal model it may take some time for the gliosing stimuli to arrive at the nucleus after damage at a distant site on the axon.

INVESTIGATION OF THE CATECHOLAMINE SYSTEM IN THE COBALT MODEL

B. Metabolites

Concentrations of HMPG (Table A5) in both ipsi- and contra-lateral cortex showed marked rises at 8 days post-implant, and appeared to have returned to normal by days 15-20. The changes in both ipsi- and contra-lateral caudate nucleus were fairly similar (Table A6) HMPG levels showing an increase at day 8 but returning to normal slightly more quickly, at day 15. Again, here is a pattern of similar changes occurring around both primary and secondary foci.

The assay used to estimate concentrations of HVA was insufficiently sensitive for a bilateral assay of striatal HVA, and samples of both lesion and contralateral side from 2-3 rats had to be pooled for analysis. The results (Table A7) indicated that HVA concentrations increased by between 50 and 100% in the mixed caudate samples between days 4 and 30 post-implant. Although one cannot be sure, the size of the increase suggests that the changes in HVA levels occurred bilaterally.

Table A7

Estimation of homovanillic acid (HVA) in caudate nuclei of rats at intervals after cobalt implantation (ng HVA/g wet weight brain)

A) Control values (Unoperated littermates)

1033
1076
814
1034
934
713

934 ± 131 (6)

B) Cobalt-implanted rats (Lesion & contralateral sides pooled)

Day 4	2677 1988 1307	Day 8	1446 1526 1574
	1991±559(3)P2		1515±53(3)P3
Day 15	784 1191	Day 20	1407 1431 1709 1410
	987(2)		1489±127(4)P3
Day 100	1134 1097 1206		
	1146±45(3)P1		

Notes:

1. All final values are given as the mean ± standard deviation.
2. To provide adequate tissue for analysis, samples of both lesion and contralateral side caudate were pooled from 2-3 rats to provide each datum included in the table.
3. P-values all refer to comparisons between control and experimental rats. Student's t-test was used, with Bessel's correction. P1 = $0.05 > P > 0.025$; P2 = $0.005 > P > 0.001$; P3 = $P < 0.001$.

Table A8

Estimation of noradrenaline (NAd) in ipsilateral cortex of cobalt-implanted rats (ng NAd/g wet weight brain)

A) Unoperated littermates (frontal cortex)	159 187 202 249 213 ± 26(4)
B) Cobalt-implanted rats (frontal cortex, lesion side)	
Day 6 post-implant	164 268 191 143 201 ± 52(4)
Day 12 post-implant	230 323 168 240 ± 64(3)
Day 30 post-implant	314 177 143 155 197 ± 68(4)

Notes:

1. All final values are given as mean ± standard deviation.

Table A9

Estimation of mid-brain tyrosine hydroxylase activity (n.mol. Dopa produced/g wet weight brain/h) in the cobalt-implanted rat

		Ipsilateral	Contralateral
A)	Control values (Unoperated littermates)	1.99	2.43
		1.30	2.43
		2.43	0.47
		2.01	0.15
		1.10	0.17
		1.06	1.64
		1.65±0.49(6)	1.22±0.93(6)
B)	Experimental animals 25 days post-implant	2.44	2.05
		1.12	2.42
		2.35	0.24
		1.04	0.21
		1.70	1.30
		1.66	2.40
		1.72 ±0.46(6)	1.44±0.99(6)
	30 days post-implant	1.48	1.99
		1.66	1.30
		2.05	2.43
		2.44	2.43
		1.12	1.06
		2.35	1.91
		1.85±0.47(6)	1.85±0.52(6)

Notes:

1. All final values are given as the mean ± standard deviation.
2. Student's t-test with Bessel's correction was used to compare the experimental data with control values. No significant changes were found ($P > 0.2$).
3. Mid-brain sections prepared as described in Methods Section, A IV.

INVESTIGATION OF THE CATECHOLAMINERGIC SYSTEM IN THE COBALT MODEL

C. Further parameters

Noradrenaline (NAd) was assayed in the ipsilateral cortex, and tyrosine hydroxylase (TOH) activity was measured in mid-brain sections containing the ipsi- and contralateral locus coeruleus. (Tables A8, A9). The finding of unchanged levels of NAd in the cortex is supported by fluorohistochemical studies of the cobalt-implanted rat in which no alteration of NAd-attributable fluorescence in the cortex was detected. (Bjorklund and Emson, personal communication). TOH activity also remained unchanged in the ipsi- and contralateral midbrain sections of the epileptic rat.

DISCUSSION

The results presented above form a rather contradictory set of data. The fact that the changes in enzyme activities and metabolite concentrations occurred in both primary and secondary foci suggested that the catecholaminergic system might be reacting at the cell body level, but the fact that no bilateral or unilateral changes in TOH activity could be demonstrated in the loci coerulei argued against this, as did the unaltered cortical NAd concentrations. This implied that the changes described above were a reflection of local cortical events rather than aspects of a physiological response in a discrete neuronal system. This is also suggested by the rate of onset of the changes in the catecholamine-related enzymes and metabolites, which resembled fairly closely the overall pattern of cellular degeneration in the cobalt model as described by Emson & Joseph (466), and also appeared to coincide with the developing changes in the EEG, although this in itself does not distinguish

between the causative or simply associative mode. If the changes in catecholamine-related parameters are not due to changes at the cell body level, then the problem arises of how to explain the rapid and dramatic changes in the secondary focus. As evidenced by the changes in the primary focus, various processes occurring within catecholaminergic nerve terminals appear to be very sensitive to cobalt. One only has to hypothesise some form of fairly rapid transport of the cobalt to the contralateral cortex to provide a putative explantation of the parallel changes occurring in the contralateral cortex. If the cobalt ion is in fact inhibiting the enzymes TOH, MAO and COMT, then the fact that decreased activities of these enzymes can be demonstrated using in vitro assay techniques where the series of dilutions involved in preparing the tissue for incubation would substantially reduce the concentration of the cobalt ion, suggests that the inhibition is of the irreversible type, and that the cobalt in the cortex, possibly concentrated in the region of catecholaminergic nerve terminals, effects a permanent alteration of the enzymes' structure. This possibility is discussed in a later section.

The increased HMPG concentrations in cortex and caudate nuclei (ipsi- and contralateral to the lesion) at day 8 can be explained in terms of only two processes, one being an increase in NAd catabolism and the other being a reduction in HMPG transport from the brain. In the light of the reduced TOH, MAO and COMT activities and unaffected cortical NAd concentrations already described, it seems most reasonable to suggest an inhibition of HMPG transport as the prime reason.

Unfortunately the assay method used in this study measured total HMPG concentrations, not distinguishing between free and conjugated HMPG (HMPG-SO_4). Free HMPG is thought to be readily diffusible throughout brain; some workers suggest that no HMPG transport system exists (Korf et al., 465) and if it can indeed diffuse so easily then teleologically there would appear to be no reason for a transport system to have developed. This theory is supported by the findings of low regional variation of HMPG; C. Yates (personal communication) finds only a two-fold maximal regional difference for total HMPG in areas of rat brain, the highest levels occurring in brainstem and the lowest in cerebellum. Ashcroft et al. (529) have published similar findings in the dog brain, with a maximal variation of 400 per cent, and similar data is being produced by the MRC Brain Metabolism unit in an ongoing project using post-mortem human brains.

This is in marked contrast to the patterns of HVA and 5HIAA distribution, which display up to ten-fold regional variation, and it is also in contrast to the pattern of localisation of the monoamine neurotransmitters. Although HMPG removal from brain is different from HVA, 5HIAA and HMPG-SO_4 excretion in that it is relatively insensitive to probenecid, a recent paper by Barany (566) has established the co-existence of two distinct transport mechanisms in the rat choroid plexus. As well as the classical, renal secretory-like system, he showed that another system, resembling that of hepatic bile acid secretion, could be demonstrated, it being less sensitive to probenecid. Although there is as yet no evidence for HMPG being a candidate for this second transport system, this

possibility cannot be discounted.

The rise in total HMPG levels could be due to an interference with the removal of HMPG-SO_4. Eccleston and Ritchie (532) have shown that HMPG is readily esterified in rat brain *in vivo*, and there is some evidence for an HMPG-SO_4 transport mechanism from the brain in the rat (527, 528), which resembles 5HIAA and HVA transport in that it can be inhibited both by probenecid and by ischaemia (527). As already pointed out, the method of HMPG assay used here does not distinguish between free and sulphated HMPG (only total HMPG was measured), so I do not know the contribution of the two species to the rise. If it is true that free HMPG is removed by diffusion, while the sulphate is removed by a transport system, the increase in total HMPG content of cortex and caudate at day 8 seems more likely to be a result of HMPG-SO_4 retention. If inhibition of HMPG-SO_4 transport were indeed a factor, then one might expect to find similar increases in HVA and 5HIAA. One of the series of 5HIAA data did in fact display an increase in cortical 5HIAA concentrations during the first week after implant; similarly, 5HIAA concentrations were found to be significantly elevated in the caudate nuclei between days 4 and 15 post-implant (Tables A18,A19). These data are discussed in more detail in the 5HT section. The HVA data also displayed a significant rise (by about 100%) in striatal concentrations at day 4 post-implant, but this elevation appeared to be longer lasting than that of HMPG, as it was still apparent at day 20. Although these changes do not exactly match, they all suggest that a block of metabolite transport was developing sometime between day 4 and up to day 20. This may

be an affect of the cobalt ion, or a result of the general trauma of the implant and subsequent epilepsy. Unfortunately the controls used in this experiment were unoperated litter-mates of the experimental animals, rather than glass-implanted, and in the absence of further data it is impossible to be more specific on this point.

It was decided to examine in more detail the possible mechanisms involved in the rapid, bilateral reduction in cortical TOH activity. The pteridine-sensitive hydroxylases are notoriously sensitive to a wide range of factors, one important one being the type of co-factor used. There is one commonly used synthetic pterin, 5, 6-dimethyltetrahydropteridine ($DMPH_4$), which has often been used in place of the physiological cofactor, tetrahydrobiopterin. Not only does the enzyme's affinity for the different cofactors vary, (Km for $DMPH_4$ is three times that for the tetrahydrobiopterin) but also when using the synthetic pterin the affinity of the enzyme for its substrate is drastically lowered, the Km value being increased twenty-fold (405). Parallel results have been published concerning the other pterin-dependent hydroxylase, tryptophan hydroxylase (17,18). Tetrahydrobropterin has also been shown to increase Vmax. values for TOH (406), and the chemical efficiency of the hydroxylation reaction (406,407), in comparison with data derived using $DMPH_4$.

The type of homogenising buffer used in the <u>in vitro</u> assay technique is also important, as TOH has been shown to be sensitive to a range of ions, notably the sulphate ion (406), which has a stimulating effect. Sulphated mucopolysaccharides, especially heparin, also stimulate the enzyme (540). Returning to the cobalt-

implanted rat, it is known that a general feature of tissue damage is an initial fall in the level of sulphated mucopolysaccharides, with a subsequent increase associated with scarring. This could complicate the picture in the primary focus, but does not seem likely to affect TOH activity measured in the secondary focus. As the results generated by the particular assay used are dependent not only on the actitity of the enzyme but also on the availability of endogenous substrate, the criticism has been raised that a fall in tyrosine hydroxylase activity could be partly caused by a fall in tyrosine content of the sampled areas of brain. However, no other amino acid assayed in this model (466) shows the decrease in concentration in both foci necessary to explain the bilateral fall in TOH activity. It is perhaps more reasonable to suggest that the cobalt ion is affecting a permanent inhibition of the enzyme, perhaps by causing some subtle alterations of the enzyme structure. The normal enzyme activity in the loci coerulei suggests that enzyme synthesis is unaffected, but possibly transport of the enzyme to the nerve terminals is inhibited. A reduction in the number of noradrenergic nerve terminals in and around both foci would also explain the reduced TOH activity, but the unchanged levels of cortical NAd argue against this, unless one postulates a compensatory increase in NAd concentration in the remaining functional neurones.

The picture then is of a greatly reduced activity of TOH in the cortex, yet normal concentrations of NAd. This seems contradictory, but can be explained by a redundancy factor - it is generally held that levels of neurotransmitter synthesising enzymes are in excess of what is required for normal functioning of the nerve, and in

particular Mackay (526) has shown that in the sympathetic ganglion preparation, no correlation whatsoever could be found between potential TOH activity and NAd concentrations. The situation as regards MAO and COMT is very different. Unlike TOH, which is restricted to a single nerve species, both MAO and COMT have been shown to be associated not only with catecholaminergic neurones, but also with non-catecholaminergic neurones and with glia (366,367). To account for the changes in these two enzymes, therefore, either one has to postulate a degree of general cell damage which, again, does not correlate at all with the unaltered cortical NAd concentrations nor with histological studies of this model (466), or one has to argue that the changes are specific. In that case one is faced with two possibilities - either the cobalt ion interferes with the synthesis of these two enzymes, or, due to a very high affinity of the ion for the enzymesand/or their environs, a finite amount of the ion continually inhibits the newly-synthesised enzymes.

The coincidence that the three enzymes should be affected in such a similar way, in contrast to, for example, gabaminergic and cholinergic enzymes, is so unlikely that common factors must be looked for. Would the fact that all three enzymes are thought to require the presence of a divalent metal ion cofactor render them especially vulnerable to the effects of the cobalt ion? (TOH requires Fe^{++}, COMT requires Mg^{++} and MAO is thought by some workers to require Fe^{++} also (602)). If these enzymes have a high affinity for cobalt, more so than, say, GABA-T, then this might explain the long-lasting changes. Alternatively, if by one of the mechanisms mentioned the cobalt ion first affects tyrosine hydroxylase activity, the changes in MAO and COMT may be compensatory phenomena, post-

and peri-synaptic responses to altering catecholaminergic tone. These longer-term phenomena would probably not be important in affecting the initial changes in enzyme levels, for example the reduced activity of MAO and COMT at day 3 in both ipsi- and contralateral cortex.

It seemed necessary at this point to check on whether the apparent reduction in tyrosine hydroxylase activity was a real phenomenon or an assay artefact. It was therefore decided to examine the effects of postulated enzyme inhibitors (including the cobalt ion) on the assay, and to measure concentrations of cobalt in the rat brain at different times after implant.

Two possible enzyme inhibitors were examined. First, it had been suggested that the cobaltous ion might compete with the ferrous ion co-factor, so the effects of the ferrous ion on cortical homogenates from cobalt-implanted rats were examined (Table A10). Enzyme activity in samples of contralateral cortex were unaltered by the addition of Fe^{++} to a final concentration of 50μM. Second, it was decided to examine the effects of HMPG, in the light of the large changes in the concentration of this metabolite in cortex and caudate during the onset of the epileptic condition (Table A5,6). This latter seemed a less likely candidate for the possible enzyme inhibitor, as HMPG concentrations have after all returned to normal long before enzyme activities recover. HMPG in a final concentration of 1mM, the maximum concentration found in the striatum in the earlier study, had no effect on tyrosine hydroxylase activity in a homogenate of contralateral cortex from a cobalt-implanted rat (Table A10). The sensitivity of the enzymes (TOH, MAO, COMT, GABA-T and tryptophan hydroxylase) to concentrations

Table A10

Effects of postulated inhibitors on tyrosine hydroxylase activity

A	Control cortex	Control cortex incubated with 1mM exogenous HMPG
Animal No.	1. 3.79	1. 3.60
	2. 3.13	2. 3.07
	3. 3.82	3. 3.96
	4. 3.24	4. 3.36
	3.5 ± 0.31	3.5 ± 0.32

B Contralateral cortex from rats 15 days post-implant

	Control incubation	Incubated with 50μM Fe^{++}
Animal No.	5. 0.52	5. 0.67
	6. 0.97	6. 0.91
	7. 0.43	7. 0.37
	8. 1.02	8. 0.88
	0.74 ± 0.26	0.71 ± 0.22

Notes:

1. All figures expressed as n.mol. Dopa produced/g protein/h.
2. All final values given as the mean ± standard deviation.
3. Fe^{++} was added as $FeCl_2$.

Table A11

Sensitivity of pterin-dependent hydroxylase enzymes to cobaltous ion concentration in vitro, tyrosine hydroxylase preparations being obtained from frontal cortex and tryptophan hydroxylase preparations from mid-brain

	Tyrosine Hydroxylase	Tryptophan Hydroxylase
A) Control data	3.0	3.5
	3.8	3.8
	4.2	4.0
	3.5	4.6
	$3.6 \pm 0.4(4)$	$4.0 \pm 0.4(4)$
B) Experimental data		
$10\mu M\ Co^{++}$	3.2	3.8
	4.0	4.1
	3.3	3.4
	$3.5 \pm 0.3(3)$	$3.7 \pm 0.3(4)$
$100\mu M\ Co^{++}$	3.0	3.2
	3.3	3.4
	3.2	2.8
	2.8	3.6
	$3.1 \pm 0.2(4)P1$	$3.2 \pm 0.3(4)P1$
$1mM\ Co^{++}$	1.4	2.4
	1.8	1.8
	1.0	2.7
	0.8	0.7
		2.4
	$1.3 \pm 0.4(4)P3$	$2.0 \pm 0.7(5)P2$

Notes:

1. Figures show n.mol. Dopa produced/g protein/h and n.mol. 5-hydroxyindoles produced/g brain/h for tyrosine and tryptophan hydroxylase respectively.
2. Final values given as $\bar{x} \pm SD'(N)$.
3. P-values refer to comparisons between experimental and control data. Student's t-test was used, with Bessel's correction. $P1 = 0.1 > P > 0.05$; $P2 = 0.05 > P > 0.025$; $P3 = 0.005 > P > 0.001$.
4. Co^{++} was added to the incubation media as the $CoCl_2$ salt, and pre-incubated for 10 m. before the enzyme reactions were begun.

Table A12

Sensitivity of enzymes of rat frontal cortex to cobaltous ion *in vitro*.

i. Monoamine oxidase (MAO

ii. Gaba transaminase (GABA-T)

iii. Catechol-0-methyltransferase (COMT)

A) Control data

GABA-T	COMT	MAO
341	442	23.8
299	378	29.3
297	403	34.0
336	369	25.6
318 ± 20(4)	398 ± 28(4)	28.3 ± 3.5(5)

B) Experimental data

(i) 10mM Co^{++}

GABA-T	COMT	MAO
273	328	25.8
294	377	33.5
358	333	27.0
311	357	22.8
309 ± 31(4)	349 ± 19(4)P2	27.3 ± 3.5(5)

(ii) 100µM Co^{++}

310	187	30.4
268	194	27.3
276	218	33.8
	144	25.0
		24.7
285 ± 18(3)P1	186 ± 27(4)P3	28.3 ± 3.5(5)

(iii) 1mM Co^{++} (MAO in presence of 3mM Co^{++})

284	29.3	41.3
304	37.1	37.9
281	35.0	42.1
	44.8	35.5
290 ± 19(3)P1	36.5 ± 5.5 P4	39.2 ± 2.7(4)

(iv) 10mM Co^{++} (MAO in presence of 30mM Co^{++})

187		16.2
214	TISSUE	16.5
178	BLANK	12.0
	VALUES	9.7
		11.4
193 ± 15(3)		13.2 ± 2.7(5)P4

Notes:

1. Figures show n.mol. GABA transaminated/g protein/h (GABA-T),n.mol. HMPG produced/g protein/h (COMT), and µ.mol. tyramine oxidised/g protein/h (MAO).
2. Final figures given as $\bar{x} \pm SD'(N)$.
3. P-values refer to comparisons between experimental and control values. Student's t-test was used, with Bessel's correction. P1 = $0.2 > P > 0.1$; P2 = $0.05 > P > 0.025$; P3 = $0.005 > P > 0.001$; P4 = $P < 0.001$.
4. Co^{++} was added to the incubation media as the $CoCl_2$ salt, and pre-incubated for 10 min. before the enzyme reactions were begun.

of the cobaltous ion was measured (Tables A11,12), and an analysis of this data is presented in the discussion of the next section, dealing with the results of the assay of cobalt in various areas of the brain.

ESTIMATION OF COBALT CONCENTRATIONS IN THE BRAIN OF COBALT-IMPLANTED RATS

It was decided to assay cobalt levels in different areas of the brain of the cobalt-implanted rat to find out whether cobalt from the implant had been transported in some way to the secondary focus and could be contributing to its development. Were any of the changes found in the cobalt-implanted rat related to an action of cobalt outside the primary focus?

Cobalt was estimated in various brain regions by flameless atomic absorptiometry (Methods DIII). The concentrations found in the various regions at different times are given in Table A13.

At day 6 after implant, the substantial levels of cobalt in ipsilateral occipital cortex and caudate indicated a lateral and downward spread of the cobalt from the implant zone. The figures for contralateral cortex and occipital cortex (means 16.8 and 15.5 ng cobalt/mg tissue respectively) give some indication of the degree of spread of the cobalt from the area of implant, and raise the possibility that the secondary focus may not be a pure mirror focus, but may be instead simply a reflection of the cobalt that has in some way reached the cortex contralateral to the site of implant.

The raphe region also contained small amounts of cobalt (4-5µg cobalt/mg tissue). It is impossible to say in this case whether this represents diffusion of the cobalt from the implant, or whether

Table A13

Cobalt (μg/g wet weight tissue) in different areas of the brain of cobalt-implanted rats

A) Control values. No cobalt could be detected in any samples from control rat brain.

B) Rats 6 days post-implantation

Rat number	1	2	3	4	5	$\bar{x}$±SD'(N)
Frontal cortex(1)	39	38	65	38	63	49±13
" " (c)	14	14	28	10	20	17±6
Occipital cortex(1)	-	10	37	19	35	25±11
" " (c)	-	4	12	19	28	15±8
Caudate nuclei(1)	34	15	30	37	38	30±8
" " (c)	10	2	17	17	30	15±9
Raphe nuclei(1)	4	1	7	1	11	4±3
" " (c)	11	2	4	7	4	5±3

C) Rats 21 days post-implantation

Rat number	7	8	9	10	11	12	$\bar{x}$±SD'(N)
Frontal cortex(1)	59	47	6	29	4	44	31±20
" " (c)	4	6	0	5	1	6	3±2
Occipital cortex(1)	-	25	5	10	10	17	13±6
" " (c)	-	4	2	6	5	6	4±1
Caudate nuclei(1)	5	23	12	8	2	12	10±6
" " (c)	5	4	2	5	2	2	3±1
Raphe nuclei(1)	1	2	5	1	4	15	4±4
" " (c)	0	2	4	1	1	9	2±3

D) Rats (4) 97 days post-implantation. No cobalt could be detected in any area of the brain.

Notes:

1. All final values are given as the mean ± standard deviation.
2. In all cases the glial capsule and any visible traces of cobalt-galatine was removed from samples of the primary lesions. However the concentrations of cobalt measured in these samples may have been attributable in part to 'depot' cobalt metal.
3. (1) and (c) refer to lesion and contralateral sides respectively.
4. The minimum amount of cobalt that could be detected when added to a samples of brain from an unoperated animals was 1.5ng added to a digest of 5mg wet weight brain.

any retrograde transport of cobalt in serotonergic fibres might have occurred. However, if cobalt in the contralateral cortex is instrumental in forming the secondary focus, the finding that sectioning the corpus callosum can, if carried out within 4 days post-implant, prevent the development of a secondary focus (517), suggests that transport of cobalt intraneuronally may be an important factor.

Using the figures of cobalt concentrations in brain, it is possible to examine the possibility of the enzymes TOH, MAO and COMT being inhibited *in vitro* in the assay procedure.

At day 6, cobalt levels in the secondary focus are around 17 µg/mg tissue. In the preparation of homogenates of brain for the enzyme assays the tissues were in effect diluted ten-fold, giving an estimated concentration of 1-2ng cobalt /µl homogenate. In the case of the assays for MAO and tyrosine hydroxylase there is a subsequent two-fold dilution, and for the assays of GABA transaminase and COMT a ten-fold dilution, giving cobalt levels under assay conditions of 100-500 pg cobalt/µl. The atomic weight of cobalt is 59; the above figures therefore represent 10µM cobalt in the tyrosine hydroxylase and MAO assays, and 2µM for the COMT and GABAT assays. These four enzymes had already been investigated as regards their sensitivity to the cobalt ion, and had all been shown to be unaffected by concentrations of the order of 2-10µM (Tables A11,A12).

This suggests that the *in vitro* enzyme assays were unaffected by the presence of the cobalt ion; but it does not exclude the possibility that the cobalt affects these and other enzymes *in vivo*, as the cobalt ion may be concentrated locally in brain to levels

that are sufficient to cause enzyme inhibition. Willmore et al. (379) and Emson and Joseph (466) have demonstrated using histochemical techniques that the regional distribution of cobalt in the brain after cobalt iontophoresis or implant is very uneven indeed. At 21 days post-implant the cobalt concentrations in all areas of brain sampled had fallen from the day 6 values by 50-75%, except in the immediate implant area where levels had not fallen as rapidly. No cobalt could be detected in any brain area at 97 days, and it seems probable that a significant proportion of the cobalt has been sequestered by the bone of the skull around the implant (580). Possibly at this late stage trace quantities of cobalt may still be leaching out into the brain and contributing to the longer-term epileptic activity.

It will be remembered that the three enzymes TOH, MAO and COMT display very similar changes in the cobalt-implanted rat. All three parameters of the central catecholaminergic system are low between 3 and 30 days post-implant, and are normal at 100 days. The sustained reduction of TOH activity in both ipsi- and contra-lateral cortex suggests that the catecholaminergic neurones do not recover within the same 30 day period as previously described for the cholinergic and gabaminergic systems (466). All the materials for reconstruction of cortical monoaminergic fibres must derive from extra-cortical nuclei. The long period before recovery could reflect a prolonged period of cell repair, or even regeneration, with the protein synthetic machinery of the cells being occupied with the manufacture of structural proteins before any significant synthesis of neuro-transmitter-related enzymes is allowed to occur. This sort of sequential programming of the syntheses of different

classes of protein (that is, structural before neuro-transmitter-related) has been described during neuronal development by Seeds (365).

An alternative explanation is that until a fairly late stage, the amount of cobalt diffusing into the brain tissue from the implant may still present a zone of cobalt concentration too toxic to permit any regrowth of noradrenergic nerve terminals; it is possible that these neurones are more sensitive in this respect than other types of neurone. There is histochemical evidence for selective cobalt adsorption by specific cell types in the cortex (notably the giant pyramidal cells, which are subsequently killed in the tissue around the primary cobalt focus (295, 466), and it has also been demonstrated that, while noradrenergic fibres regrow into freeze-lesion foci (467), they will not regrow into cobalt foci (Bjorklund and Emson, personal communication).

A third explanation for the long-delayed recovery would be related to rates of axonal regrowth. The time course of axon regrowth has been shown by various workers to be of the order of 75-100 days, for example the re-innervation of the tongue after hypoglossal axotomy (364). Although the times fit, this is hardly enough in itself to suggest an axonal regrowth and regeneration of cortical innervation as a basis for the biochemical findings. There is to date no evidence of cobrect re-innervation, or fibre regrowth over any distince, in the CNS of the adult mammal (368,369).

Another problem is how to distinguish an increase in monoamine-related enzymes due to re-innervation from that due to a compensatory mechanism in the remaining intact neurones. For example,

reserpine-induced monoamine depletion, and cold stress, have been reportdd to cause increases in tyrosine hydroxylase activity in the stellate and superior cervical ganglia. If the stress of cortical injury and the epileptic process affect central catecholaminergic neurones similarly, this might contribute to the apparent normalisation of enzyme activity at 75-100 days post-implantation.

EFFECTS OF AN INHIBITION OF CEREBRAL PROTEIN SYNTHESIS ON THE DEVELOPMENT OF THE SECONDARY FOCUS

The fact that cobalt could have contributed to the formation of the secondary focus rekindled the question of whether the contralateral cortex becoming epileptic was due to a transfer of the original epileptogen (in this case cobalt) or whether the secondary focus "learns" to mimic the electrical activity of the primary focus, a process involving perhaps the formation of new connections between the involved areas of cortex. In the case of other epilepsies, that following 'freeze' lesions for example, it seems unreasonable to postulate the synthesis of an epileptogen in the primary focus and its subsequent transfer to the secondary. To resolve this point it was decided to examine the effects of a protein synthesis inhibitor on the development of the secondary focus, as it has been demonstrated (596) that this type of drug can block part of the learning process. The drug of choice for this experiment seemed to be acetoxycycloheximide, a potent inhibitor of protein synthesis and, unlike puromycin, a drug free from any gross effects on the EEG (596).

The drug's effect on protein synthesis was first examined, (Methods D,Ib), and then a series of rats was given gobalt implants, treated with the drug, and recorded over a period of 3 weeks.

Table A14

Effect of acetoxycycloheximide on rates of cerebral protein synthesis in rat frontal cortex.

Control		Acetoxycycloheximide-treated	
Left	Right	Left	Right
1. 0.84	0.89	4. 0.67	0.63
2. 0.81	0.87	5. 0.64	0.53
3. 0.79	0.92	6. 0.60	0.55
0.86±0.04		0.63±0.03	0.57±0.04

Notes:

1. Each figure represents a ratio derived from TCA-insoluble (protein) counts to TCA-soluble (amino acid) counts.
2. Acetoxycycloheximide-treated animals were given an intracerebral injection of 20 µg of the drug, made up in 10 µl mammalian Ringers solution, into the right frontal cortex 48h prior to killing.
3. Each animal was given a subcutaneous injection of 0.5 ml 0.9% NaCl containing 1 µCi of a ^{14}C-labelled protein hydrolysate 1h. prior to killing.
4. All final values are given as the mean ± standard deviation.

Table A15

Effects of an intracerebral injection of 20 µg acetoxycycloheximide into the contralateral frontal cortex of a cobalt-implanted rat on the rates of spike formation/min. in a 10 minute ECoG recording.

	Days post-implant			
	6	12	16	20
A	3/2	12/53	3/38	3/11
B	0/2	12/12	30/5	8/6
C	1/1	3/0	1/2	DIED
D	0/6	20/38	6/37	9/33
E	4/0	61/81	15/159	12/105
F	0/1	0/5	DIED	
G	54/55	2/3	N.R.	0/0
H	23/25	32/31	N.R.	0/0
I	5/7	9/4	7/3	5/6
J	3/32	6/25	5/25	3/25
	9.4±16.8/13.6±17.5	16.7±17.6/25.2±25.0	9.6±9.3/38.4±51.3	6.7±3.3/31.0±34.6

Notes:

1. x/y represents spikes in primary/secondary epileptic foci.
2. N.R. indicates no recording was made.
3. 20 µg of acetoxycycloheximide was given at days 1, 3 and 5 post-implantation.
4. All final values given as the mean ± standard deviation.

Acetoxycycloteximide has a long duration of action, reducing rates of cerebral protein synthesis by around 55% at up to 2 days after administration of the drug (Table A14). The cobalt-implanted rats were given the drug at days 1, 3 and 5 post-implant, to maintain a degree of inhibition of protein synthesis over the first week. The results (Table A15) suggest that a 55% inhibition of cerebral protein synthesis may in some cases be associated with subnormal rates of spiking in both foci at day 6 post-implantation. These results are however difficult to interpret, because the effect was not consistent, a significant minority of the experimental animals displaying normal spiking rates in both foci.

As this degree (55%) of cerebral protein synthesis inhibition is similar to that found by Barondes and Cohen (596) to interfere with the learning process, it was felt that, while various types of protein synthesis may be associated with the development of the epileptic foci, the question of the causation of the secondary focus could be answered most satisfactorily in this model by postulating the transfer of cobalt as the critical factor.

THE ROLE OF THE 5-HYDROXYTRYPTAMINERGIC (5-HT) NEURONAL SYSTEMS IN THE COBALT-IMPLANTED RAT

After the studies of the catecholamine-related enzymes and metabolites had been completed, a further series of experiments was undertaken to investigate the involvement of the serotonergic systems in the cobalt model.

It would be appropriate here to discuss first the evidence for 5HT as a central neurotransmitter, and second the work that suggests that the 5HT systems are involved in certain states of central nervous system malfunction, specifically the epilepsies.

Much detailed biochemistry has been done on animal models; however, the situation in man is obviously less accessible to study, and most clinical investigations are limited to the analysis of urine, blood and CSF samples. In order to interpret the clinical data in the light of the structure of evidence provided by animal models, the functioning of the 5HT systems in the control and model animal must first be clarified.

In the following section a brief summary of the present knowledge of the role and function of central 5HT is presented, including evidence supporting the three criteria of postulated neurotransmitters: namely the appropriate presence or location of the compound and its synthetic and catabolic enzymes, its release on stimulation of the appropriate nerves, the ability of the exogeneous compound when appropriately administered to mimic the effects of the physiological release of the endogenous compound, and finally, the ability of the exogenous compound to mimic the endogenous compound in terms of its activity being appropriately modified by drugs known to act on the endogenous system.

THE EFFECTS OF EXOGENOUS 5HT

A series of micro-iontophoretic studies has been carried out to examine the action of 5HT neurones in the brain. The general consensus is that 5HT is primarily inhibitory in effect, although cells in certain areas of the brain have been demonstrated to be excited by 5HT.

In the cortex, Roberts and Straughan (178) reported that iontophoresed 5HT inhibits one third of cortical cells, excites another third and is inactive on a further third. This study also investigated the interactions between 5HT and other drugs, establishing that the excitatory action of 5HT could be blocked by methysergide, a drug known to block the peripheral actions of 5HT. The inhibitory action of 5HT could not be blocked, however. This was later verified by Krjnevic and Phillis (482) who, however queried the findings of 5HTs excitatory action, and suggested that 5HT in the cortex was almost entirely inhibitory in function. Jordan et al. (483) published a paper in 1972 suggesting that the excitatory action of 5HT in the cortex described by Roberts and Stranghan (178) and others was in fact artefactual, caused by pH effects rather than the 5HT. The picture is by no means clear, though, because recently a report by Boakes et al. (129) has described how the excitatory action of 5HT on cortical neurones can be blocked using lysergic acid diethylamide (LSD) and 2-bromo-d-lysergic acid diethylamide (BOL-148), drugs thought to act as 5HT receptor blockers. This would support the idea that the excitatory action of the iontophoresed 5HT is a physiological response rather than an artefactual one.

In areas of heavy serotonergic innervation, including the optic tectum, amygdala and ventral lateral geniculate, iontophoresed 5HT has an inhibitory effect (484, 485). In the raphe nuclei too, 5HT has been demonstrated to have an inhibitory action and it has been suggested that this may indicate the presence of an auto-inhibitory modulating mechanism (484). Cells in the reticular formation which may be involved in the postulated reticular activating system, fall into two classes, some being excited and others inhibited by 5HT (486).

CRITERION OF LOCATION

Fluorohistochemical work by Dahlstrom and Fuxe (168) showed 5HT in the rat brain to be located solely in nerve cells whose bodies lie in the mid-brain and brain-stem, primarily in the raphe nuclei. The cells in the rostral raphe nuclei project mainly to the forebrain, and the caudal nuclei mainly to the spinal cord (168, 169), in a pattern similar to that found in other animals (170). Concentrations of 5HT appeared to be greatest in the nerve terminals, and very much lower in the cell bodies and axons (168,171). Sub-cellular fractionation studies also showed that a substantial proportion of 5HT (and NAd) in brain was to be found in the synaptosomal fraction (195, 196), associated with a population of dense-cored vesicles (196,198). Autoradiographic work by Aghajanian and Bloom (197) supplied corroborative evidence, finding that tritium-labelled 5HT given intraventricularly was mostly taken up by nerve-terminals, primarily those containing dense-cored vesicles. These dense-cored vesicles, found in the varicosities of terminal axons and not in the inter-varicosal segments, (195), are thought to be involved in the release of the neurotransmitters NAd and 5HT (176,198).

The effects of electrical stimulation of the raphe were first investigated by Aghajanian et al. (201, 202), who found that this treatment resulted in an increase in 5-hydroxyindole acetic acid (5-HIAA), the principal metabolite of 5HT together with a fall of 5HT in the fore brain. Unilateral surgical lesioning of the medium fore-brain bundle prevented these changes on the affected side; at longer periods after lesioning, ipsilateral cortical 5HT and 5HIAA levels fell, reflecting the degeneration of the serotonergic nerve terminals (203-208). Raphe stimulation has been shown to increase 5-HIAA release into cortical perfusate in the rat, and into cat CSF (209, 210). As all these studies (203-210) with one exception (201) found that stimulating the serotonergic system increased 5-HIAA formation without affecting 5-HT concentrations, it seems that such stimulation increases 5-HT synthesis in such a controlled manner that 5-HT concentrations remain constant.

More recently an elegant single-cell recording study by Bloom et al. (552) has indicated that the effects of raphe stimulation are uniformly inhibitory on all identified post-synaptic cells, and the authors suggestdd that the raphe has a general tonic inhibitory influence on all the cells to which it projects. These observations indicate again that the studies showing an excitatory effect of 5HT did not reflect the physiological situation.

SYNTHESIS OF 5HT IN BRAIN

Metabolism: The synthetic pathway of 5HT is illustrated in Figure A4.

The amine itself is thought not to pass the blood brain barrier in significant amounts (Axelrod and Inscoe, 179), but recently it has been demonstrated that traces of 5HT given intravenously do in fact

enter the brain, although this is only of significance in the first ten minutes after injection (587) because of the rapid clearance of 5HT from the blood, and equally rapid oxidation of 5HT in the brain. It is generally assumed, therefore that any 5HT found in brain has been synthesized *in situ* from a precursor in the blood, either tryptophan or 5-hydroxytryptophan (5HTP). Normally 5HTP cannot be detected in the blood (180), so the sequence of synthesis is thought to commence with the uptake of tryptophan from the blood into the brain, with all subsequent synthetic steps occurring in the brain.

Tryptophan hydroxylase activity has been demonstrated in brain by various workers, including Grahame-Smith, 1964 (181), Green and Sawyer, 1966 (182), Ichiyama *et al.*, 1968 (183) and Gal *et al.*, 1963 (184). It has been shown by Graham-Smith (480) and others (185,475) that the distribution of this enzyme parallels the distributions of 5HT and of 5HT nerve terminals as described by Dahlstrom and Fuxe (168). Sub-cellular studies (183, 480) have found that, in rabbit hind-brain, around half of the tryptophan hydroxylase activity was associated with the synaptosomal fraction.

The second step in the synthesis of 5HT is the decarboxylation of 5-HTP; the enzyme responsible is thought to be the relatively unspecific L-aromatic amino acid decarboxylase (476); immunohistochemically the decarboxylase in serotonergic neurones behaves identically to the unspecific decarboxylase, which is widely distributed throughout the brain (476).

The rate-limiting step *in vitro* (in brain homogenates, 183) and *in vivo* (in dog and rat brain, 187) appears to be the initial hydroxylation. The K_M of the hydroxylase for its substrate, tryptophan is reported to be of the order of 50μM, a value closely in line with

published values for brain tryptophan concentrations (17,18). It is thought that the rate of tryptophan hydroxylation is highly sensitive to substrate levels in brain, and hence ultimately influenced by plasma free tryptophan concentrations (180,188,190,199).

Theoretically the order of the two synthetic steps could be reversed, but the affinities of the two enzymes for the respective substrates rules against that possibility as a major pathway (183). However, Marsden and Curzon (478) and others (479) have demonstrated endogenous tryptamine in rat brain, and have also shown that a proportion of brain tryptamine is formed in serotonergic neurones. Other workers suggest that the hydroxylase and the decarboxylase are in some way coupled; an *in vitro* synthesis of C^{14}-5HT from C^{14}-tryptophan was reported to be so efficient (183) that no trace of C^{14} 5HTP could be found in the reaction medium containing unlabelled carrier 5HTP, indicating that the labelled intermediate was taken up by the decarboxylase immediately after hydroxylation.

Catabolism

5HT is oxidised by monoamine oxidase (MAO), forming 5-hydroxyindole acetaldehyde. This intermediate is further oxidised by aldehyde dehydrogenase to form 5HIAA, 5-hydroxyindole acetic acid. An alcohol dehydrogenase has also been demonstrated in brain, and a small proportion of the 5-hydroxyindole acetaldehyde is metabolised to 5-hydroxytryptophol (192, 193). It is also theoretically possible for 5HT to be removed from brain as a conjugated sulphate; however Korf and Sebens (194) were unable to detect endogenous 5HT-O-sulphate in rat brain, and Eccleston and Ritchie (532) have shown that 5HT is not a substrate for the sulphotransferase system in rat brain, although the tryptophol might be.

Regulation of 5HT synthesis

A regulation of tryptophan hydroxylase activity was first hinted at in studies of the rates of 5HT synthesis in animals treated with drugs to raise or lower central levels of 5HT. Tozer et al. (285) demonstrated an increased rate of 5HT synthesis in reserpinised rats, and, conversely, mice pre-treated with monoamine oxidase inhibitors appeared to have subnormal rates of central 5HT synthesis (587).

Various experimenters attempted to study the phenomenon of an end-product feed-back system in an in vitro situation, looking for a reduction in tryptophan hydroxylase activity in preparations of mid-brain when incubated with high concentrations of 5HT. McGear and Peters (287) published negative results; and other studies (288, 289) failed to demonstrate any inhibition of tryptophan uptake into brain synaptosome preparations. Meek and Fuxe (290, 435, 436) and others (291) found that after MAO inhibition, the concentrations of brain 5HT rose much more than those of central catecholamines and attributed this to the lack of a serotonergic end-product inhibition but Glowinski et al. (292, 293) reported that when 5HT levels reached three times normal, after MAO inhibition, a reduction of 5HT synthesis did occur. They suggested that this regulation developed at the hydroxylase level, since the synthesis inhibition did not affect the conversion of ^{3}H-5HTP to ^{3}H-5HT. Hamon et al. (294) used brain slice preparations from rat striatum to demonstrate that a high concentration of 5HT in the incubating medium did inhibit the rate of 5HT synthesis, as measured by 5HT and 5HIAA formation from ^{3}H-tryptophan. In this elegant experiment the inhibitory effect depended on intraneuronal 5HT levels, as the inhibitory effect of 5HT was lost after chlorimipramine,

of 5HT neuronal uptake blocker was added to the medium. Tryptophan uptake was not affected by the high 5HT concentrations.

In summary, it would appear that an end-product inhibition mechanism does exist, although it appears to be less sensitive than the system operating in catecholaminergic neurones (292,293).

ANALYSIS OF THE PARAMETERS TO BE USED IN THE INVESTIGATION OF THE COBALT RAT

It was necessary to decide which of all the available parameters of the serotonergic system would provide the most data on the cobalt model. Those parameters chosen should preferably reflect central events only, and remain unaffected by changes outwith the C.N.S. Rates of tryptophan hydroxylation centrally, for example, are thought to be influenced by plasma free tryptophan concentrations (180,188-190) amongst other factors. This can be allowed for, under laboratory conditions, by using a genetically uniform strain of animals in a standardised environment, fed on a uniform diet and experimented on at set times of the day.

Systemic changes in 5HT metabolism, on the other hand, do not affect the central levels of 5HT and 5HIAA, as these compounds do not cross the blood-brain barrier.

Concentrations of 5HT in the brain are not a good index of central 5HT turnover. Electrical stimulation of the raphe, as already noted (203-210) results in increased 5HT turnover unaccompanied by any change in 5HT concentrations. In the reserpinised rat, 5HT turnover increases while 5HT levels are low (285).

In this respect an assay of 5HIAA provides more information. 5HIAA levels depend on two factors, one being the rate of oxidative

deamination of 5HT and the second the rate of removal of the metabolite from the brain into the CSF and blood. There is some degree of passive diffusion of 5HIAA from the brain, and there also exists an excretory mechanism (213,214), which has been shown to resemble the excretion of 5HIAA from the CSF to the blood in the dog (215) and the renal tubular secretion of organic acids in the kidney (215). Perez-Cruet et al. (216), charting the rate of 5HIAA disappearance from brain after MAO inhibition, found that it was proportional to the levels of 5HIAA in the brain. This linearity was lost at concentrations in excess of 180 per cent of normal when the transport mechanism became saturated, and a change of slope indicated that farther increases in the rate of 5HIAA removal could only be achieved by diffusion. If 5HIAA efflux from brain is normally proportional to its concentration, then continuous monitoring of cerebral 5HIAA concentrations would provide an excellent indication of rates of 5HT turnover. A single estimate does not furnish as much information, as a datum so obtained can only reflect 5HT turnover in the steady state condition. And it may well be that the steady state is a vanishingly rare phenomenon. Work by Knapp and Mandell (218 and 231, 232, 234) shows that the administration of many, if not all, drugs that act on the metabolism of aminergic neurones results in a prolonged series of adaptative responses by the neurones to the new conditions of function, rather than in a new steady state. Levels of 5HIAA may be used as an approximate measure of 5HT turnover in a non-steady state condition with more validity if 5HT concentrations are also measured, as one can then view the 5HIAA data in the light of the nerve terminals' capacity to store 5HT. This might be of considerable

importance of 5HT displacement in conditions, where measurements of 5HIAA concentrations could give a misleadingly high indication of 5HT turnover.

Cortical 5HT and 5HIAA concentrations, and rates of cortical 5HT uptake, were therefore selected as an index of the functional state of the serotonergic nerve-terminals, and tryptophan hydroxylase was assayed in the anterior raphe nuclei to provide information on the state of affairs in the cell bodies. All the assays were fluorimetric; 5HT and 5HIAA were measured using the techniques of Schlumpf et al. (24), Methods CV and the extraction procedure of Roos (211), Methods CII. The tryptophan hydroxylase assay used was that of Baumgarten et al. (243), Methods BII: it was insufficiently sensitive to measure enzyme activity in the cortex.

ALTERNATIVE METHODS OF MEASUREMENT OF 5HT TURNOVER

1. Accumulation of 5HIAA in brain after inhibition of the metabolite transport system

The drug generally used is probenecid. It effectively inhibits the active transport of 5HIAA from rat brain (214), and from CSF, in the dog (214). After probenecid administration to rats, 5HIAA has been found to accumulate linearly for up to 8 hours, reaching 6 times the normal level (598), and the drug has also been shown to almost completely halt the decline in 5HIAA after MAO inhibition (214). This means that the active transport system must account for nearly all the removal of 5HIAA from brain. In addition, the fact that 5HIAA levels continue to rise linearly even when grossly above normal (598) suggests that the build-up of 5HIAA does not exert a feedback inhibition on 5HT synthesis, a possibility which might otherwise cast

doubt on the validity of this method of measuring 5HT turnover.

Probenecid itself does not appear to affect the rate of tryptophan hydroxylation, as the rates of 5HT turnover calculated in this manner agree with the results calculated by measuring rates of removal of 5HIAA after MAO inhibition (214).

2. Rate of removal of 5HIAA from brain after MAO inhibition

This method is based on the fact that removal of 5HIAA from the brain is directly proportional, over a certain range, to its concentration (216). As already pointed out, however, at elevated 5HIAA levels saturation of the transport mechanism occurs, and the relationship between 5HIAA concentration and rates of 5HIAA efflux alters. Saturation of the transport mechanism in the rat is thought to develop when 5HIAA levels reach 180% of normal values (216). One must also make the assumption that the metabolite transport system is not affected by the MAO inhibitor, and that 5HIAA is the sole metabolite of 5HT; this latter is probably not completely true, as there is evidence of some formation of 5-hydroxytryptophol *in vivo* (D. Eccleston, personal communication), but the amounts of 5HT thus metabolised are insignificant.

3. Accumulation of 5HT after MAO inhibition

This method has also been used to measure cerebral 5HT turnover, and has given results similar to those obtained by the other methods (212). It assumes that 5HT is only catabolised by MAO, that a total inhibition of MAO can be achieved, and that no 5HT is lost from the brain by diffusion. It also assumes that the inhibitor used does not affect tryptophan hydroxylase directly, and that the increasing levels of 5HT do not exert a product-inhibition effect either.

In fact this last assumption is not completely true, as there is evidence of inhibition of brain tryptophan hydroxylase by high concentrations of 5HT both *in vitro* (294) and *in vivo* (212); however, calculations of 5HT turnover based on initial rates of 5HT accumulation after MAO inhibition give data in line with the results obtained using other methods (212).

4. Increases in brain 5HT and 5HIAA after tryptophan loading

This method involves giving the experimental animals large doses of L-tryptophan so that cerebral tryptophan hydroxylase becomes saturated, and mesuring the subsequent changes in 5HT and 5HIAA in the brain. The technique does not give an exact quantitative estimate of 5HT synthesis, as 5HT and 5HIAA are turning over all the time; but it does provide an overview of the whole metabolic pathway, indicating whether, in an experimental situation, synthesis or breakdown of 5HT is affected or 5HIAA excretion. The cerebral distribution of 5HT and 5HIAA is the same after tryptophan loading as under normal conditions (187), although the high concentrations reached (up to 10 times normal in rat brain after doses of 400-800 mg/kg (180, 236) may trigger off a number of side-effects. Aghajanian (237) reported a reduction in cell firing rates in the raphe, and suggested that this was due to increased 5HT in serotonergic nerve terminals acting at post-synaptic receptor sites with a consequent compensatory reduction of raphe activity. He found, however, that this raphe inhibition could not be prevented by the use of a tryptophan hydroxylase inhibitor (p-chlorophenylalanine), indicating that high concentrations of tryptophan may also inhibit serotonergic neurones (237). High concentrations of 5HT

have been shown to inhibit tryptophan hydroxylase, in rat brain slices (294), and this phenomenon may also occur *in vivo*.

5. Methods using radioactive tracer compounds

5HT turnover has been measured by charting the rate of disappearance of labelled 5HT from brain after intra-ventricular administration (599), and the rate of labelled 5HT formation after intravenous administration of radioactive tryptophan either in an infusion (600) or a single injection (601). The problem with the first of these methods is that one cannot be sure whether 5HT given intraventricularly is distributed and metabolised in the same way as the endogenous amine. The other two methods also have their disadvantages, and are not widely used. The infusion technique requires that the animals be immobilised, a stress factor which could complicate the biochemical picture, and it has been found that, with the single injection technique, very slight variations in the technical procedure are capable of producing an unacceptably wide variation in results (601).

CHOICE OF METHODOLOGY

When the investigations into the role of the serotonergic system in the cobalt-implanted rat were begun, it was decided to simply measure 5HT and 5HIAA in various brain areas, and then examine the effects of tryptophan loading on the 5HT and 5HIAA levels. It was felt that, in view of the involvement of MAO in the cobalt-implanted rat as already described (Table A4) and the possibility of a block of metabolite transport after cobalt implantation (p 85), it might not be wise to use methods of measuring 5HT turnover using MAO inhibitors or probenecid.

Table A16

Estimation of 5-hydroxytryptamine (5HT) (μg 5-HT/g wet weight tissue) in cortex around primary lesion at intervals after cobalt implantation

A) **Control values ('Sham-operated' littermates)**

0.37
0.51
0.51
0.39
0.45±0.07 (4)

(B) **Cobalt-implanted rats**

Day 10	Day 20	Day 30	Day 84
0.75	0.34	0.50	0.49
0.55	0.66	0.49	0.35
0.36	0.37	0.40	0.76
0.48	0.34	0.70	0.44
0.45	0.62	0.60	0.42
0.48	0.55	0.30	0.73
		0.38	
0.51±0.12(6)	0.48±0.13(6)	0.48±0.13(7)	0.53±0.16(6)

Notes:

1. All final values are given as the mean ± standard deviation.
2. None of the experimental data differ from the control results, ($P > 0.2$); Student's t-test with Bessel's correction was used.

In addition to the above, the following parameters of the serotonergic system were also studied. Rates of 5HT uptake in the cortex were measured, to give some idea of the number and viability of serotonergic nerve terminals in samples of primary and secondary focus, and other cortical areas. Tryptophan hydroxylase activity was measured in mid-brain sections containing the anterior raphe nuclei to give some indication of the involvement of the serotonergic system at cell-body level (unfortunately the assay used proved insufficiently sensitive to measure cortical tryptophan hydroxylase activity). The concentrations of tryptophan in the cortex were also measured to examine further the possibility of alterations of tryptophan metabolism in the cobalt-implanted rat. Finally, in view of the results provided by these investigations, the effects of a series of drugs known to act on the metabolism of 5HT was examined in terms of their effects on the ECog of the epileptic rat.

THE INVOLVEMENT OF THE SEROTONERGIC SYSTEM IN THE COBALT-IMPLANTED RAT

The measurement of 5HT and 5HIAA in the cortex and striatum was carried out using the fluoroassay of Schlumpf et al. (24, Methods CV). The concentration of 5HT in the cortex around the primary lesion was apparently unaffected (Table A16). This was basically in agreement with the findings of an unaltered intensity of 5HT-attributable fluorescence in the cortex of the cobalt-implanted rat (Björklund and Emson, personal communication). However the concentration of 5HIAA in the same area of cortex showed a fall of about 25% developing between days 10 and 20 post-implant (Table A17). The time- course of this reduction appeared to be of significance, because, unlike any other parameter studied so far in the cobalt-implanted rat, it did not develop in step with the onset of non-specific cell

Table A17

Estimation of 5-hydroxyindolacetic acid (5HIAA) in cortex around the primary lesion at intervals after cobalt implantation

A) Control values ('Sham-operated' littermates)

0.37
0.43
0.44
0.35
0.40±0.04(4)

B) Cobalt-implanted rats

Day 5	Day 10	Day 20	Day 30	Day 84
0.43	0.41	0.30	0.34	0.34
0.32	0.31	0.25	0.26	0.46
0.37	0.42	0.32	0.37	0.32
0.25	0.42	0.19	0.25	0.41
0.39	0.38	0.31	0.25	0.40
0.41	0.37	0.23	0.25	0.41
0.43	0.43	0.28	0.33	0.37
0.36	0.31	0.25	0.26	0.30
0.37±0.06(8)	0.38±0.05(8)	0.27±0.04(8)	0.29±0.05(8)	0.38±0.05(8)
		P2	P1	

Notes:

1. All figures refer to µg 5HIAA/g wet weight brain.
2. Final values are expressed as $\bar{x} \pm$ SD' (N).
3. P-values refer to comparisons between control and experimental data. Student's t-test was used, with Bessel's correction. P1 = $0.005 > P > 0.001$; P2 = $P < 0.001$.
4. Assay method no. 2 was used to generate these data. Although 5HIAA levels thus measured appear slightly lower than those in table I, internal consistency is unaffected (Methods CII).

Table A18

Estimation of 5-hydroxyindolacetic acid (μg 5-HIAA/g wet weight brain) in frontal cortex of cobalt-implanted rats

	Lesion	Contralateral
A) Control values (glass implants)	0.36	0.39
	0.33	0.37
	0.39	0.29
Pooled data gives:	0.35 ± 0.04 (6)	
B) Cobalt-implanted rats	Lesion	Contralateral
Day 4 post-implant	0.41	0.50
	0.42	0.42
	0.48	0.34
	0.44±0.03(3)P3	0.42±0.07(3)P1
Day 8 post-implant	0.38	0.54
	0.33	0.35
	0.36(2)	0.44(2)
Day 15 post-implant	0.13	0.38
	0.11	0.35
	0.12(2)	0.37(2)
Day 24 post-implant	0.12	0.17
	0.09	0.16
	0.13	0.16
	0.11±0.01 P4	0.16±0.06 P4
Day 100 post-implant	0.43	0.49
	0.42	0.41
	0.39	0.31
	0.41±0.02(2)P2	0.40±0.07(3)

Notes:

1. Final values given as $\bar{x} \pm SD'$ (N).
2. P-values refer to comparisons between control and experimental data. Student's t-test with Bessel's correction was used.
   P1 = $0.2 > P > 0.1$; P2 = $0.1 > P > 0.05$; P3 = $0.02 > P > 0.01$;
   P4 = $P < 0.001$
3. Assay method no. 2 was used to generate these data (Methods CII).

Table A19

Estimation of 5-hydroxyindoleacetic acid (5HIAA) (μg 5-HIAA/g wet weight brain) in caudate nuclei from cobalt-implanted rats

A) Control values (glass-implanted littermates)

0.76
0.46
0.45
0.41
0.52±0.13 (4)

B) Cobalt-implanted rats

Day 4	Day 8	Day 15
0.82	0.74	0.75
0.73	0.73	1.39
0.70	0.71	0.91
0.75±0.05(3) P1	0.73±0.01 P1	0.98±0.12(3) P2

Day 24	Day 100
0.66	0.62
0.51	0.50
0.58(2)	0.56(2)

Notes:

1. Final values given as $\bar{x} \pm$ SD'(N).
2. Lesion and contralateral side caudate nuclei from 2-3 rats were pooled to provide sufficient tissue for assay.
3. P-values refer to comparisons between control and experimental data. Student's t-test with Bessel's correction was used P1 = $0.1 > P > 0.05$: P2 = $0.05 > P > 0.025$.
4. Assay method no. 2 was used to generate these data (Methods CII).

damage (466), or the appearance of the primary and secondary epileptic foci (466).

To follow up this experiment 5HIAA concentrations were determined in a second series of rats in ipsi- and contralateral cortex, and in the caudate nuclei. It was decided to use a slightly different assay method, this time using a separate extraction procedure (562, Methods C,III). This second series of data also showed that the concentrations of 5HIAA fell in both ipsilateral and contralateral cortex with a maximum decrease of 50-60%, probably between days 15 and 24 post-implant (Table A18). The reduction of 5HIAA in the secondary focal area appeared to develop more slowly than in the primary focus, but in view of the small number of animals assayed the significance of this was not clear. This greater reduction (50-60%) of cortical 5HIAA may have reflected the severity of the tissue reaction of the cortex to the cobalt implant; different groups of implanted rats did show variation in this respect, with the lesion in some cases being confined to the cortex around the implant and in other cases presenting with substantial erosion of the striatum below the cortical implant. This particular group developed fairly severe lesions, and the small number of experimental animals sampled was due to the number of post-operative deaths in the group.

Changes in 5HIAA concentrations in pooled samples of ipsi- and contralateral caudate did not parallel the changes in cortical 5HIAA (Table A19). In fact, in complete contrast, 5HIAA concentrations showed an increase of up to 50% from day 4 to 15 post-implant before returning to normal values. A different type of test was initiated to help to clarify these observations. Using the method of Bjorklund

Table 20

Measurement of 5-HT uptake *in vitro* into cortex of rats given cobalt implants 20 days previously (Figures given as ratio of tissue counts to medium counts, x 100. Methods DII).

A) Control values (glass-implanted littermates)

Animal no.	1	2	3	4	$\bar{x}$±SD'
Frontal (left)	499	472	390	457	454±40
Frontal (right)	448	387	334	314	370±51
Occipital (left)	380	326	474	370	387±53
Occipital (right)	353	473	366	416	402±47

B) Cobalt-implanted rats

Animal no.	5	6	7	8	9	10
Frontal (Ipsilateral)	312	451	460	467	320	447
Frontal (contralateral)	430	336	325	359	318	349
Occipital(ipsilateral)	318	359	475	361	365	305
Occipital(contralateral)	377	452	394	444	312	383

Frontal (i)	409±66
Frontal (c)	352±37
Occipital (i)	363±54
Occipital (c)	393±46

C) 0°C controls

Animal no.	11	12	13	$\bar{x}$±SD'
Frontal (ipsilateral)	53	87	58	66±14
Frontal (contralateral)	62	76	74	70±6
Occipital (ipsilateral)	83	69	55	69±11
Occipital (contralateral)	55	70	57	60±6

Notes:

1. Experimental values did not differ significantly from control values ($P > 0.25$), as measured using Student's t-test with Bessel's correction.

et al. (304, Methods DII), rates of 5HT uptake into various areas of cortex were measured. The rate of 5HT uptake into a brain sample is thought to be an indication of the number and viability of serotonergic nerve terminals in the sample (304). Rates of 5HT uptake were measured in rats 20 days post-implant in areas of ipsi- and contralateral frontal and occipital cortex (Table A20). This was at a stage when cortical 5HIAA had apparently fallen (Tables A17, A18), yet experimental values for 5HT uptake rates did not differ from control values. This finding, taken together with that of the unaltered 5HT concentrations in the cortex, suggested that there was little damage to serotonergic nerve terminals in the affected areas of the cortex, and that the changes in 5HIAA concentrations might therefore be due to changes in rates of 5HT turnover and/or rates of 5HIAA transport, rather than a reflection of the breakdown of serotonergic nerve terminals with subsequent increases in 5HT release and oxidation.

In experimental situations where marked degeneration of cortical serotonergic nerve terminals is known to occur, for example after lesioning the median fore-brain bundle, concentrations of both 5HT and 5HIAA are significantly reduced (203,204,205).

It seemed more feasible to postulate some degree of inhibition of the 5HIAA transport mechanism. The increased striatal levels of 5HIAA at days 4 to 15 resembled the increases found in cortical and striatal HMPG levels between days 8 and 15, and the increased striatal HVA concentrations found between days 4 and 20 (Tables A5,6,7). But it was difficult to reconcile the idea of a reduction of metabolite transport with the findings of reduced cortical 5HIAA until it was

realised that the reduction of cortical 5HIAA only developed between days 15 and 24, that is, just after the apparent cessation of the inhibition of metabolite transport. This complicates the picture, because having made the assumption on the basis of cortical 5HIAA levels that cortical 5HT turnover was reduced after 15 days, the apparently normal 5HIAA levels before day 15 may have concealed a combination of reduced 5HT turnover, together with an inhibition of 5HIAA transport, the latter as we have seen possibly developing by days 4-8.

Why should 5HIAA concentrations in cortex and striatum have changed in such different ways? How are these data reconcilable? One possible explanation is that the serotonergic system differentiates in its innervation to cortex and striatum, and is capable of exerting a different degree of tone on the two innervated areas. What is known about the anatomy of the serotonergic system does not actually rule this possibility out, as the cortical and striatal serotonergic innervations develops from two distinct tracts (552), not is there any a priori reason for thinking that the raphe nuclei should act in parallel.

Recently it has been shown that the central dopaminergic systems in this model may be comparably involved. Eccleston (D. Eccleston, personal communication) has found a rise in striatal HVA after cobalt implant, in step with a fall in HVA in the substantia nigra. One possible explanation might be to postulate the existence of an intermediate group of inhibitory neurones, operating so that a reduction of tone in one system induces, via the inhibitory interneurones, a local increase in activity in the other system. Could

Table A21

L-tryptophan (μg/g wet weight) in the occipital cortex of cobalt-implanted rats

A) Control values ('Sham-operated' rats)

12.2
12.8
18.4
23.6

16.8±4.6(4)

B) Cobalt-implanted rats (ipsi- and contralateral occipital cortex pooled)

Day 8 post implant:	Day 30 post-implant
9.9	60
9.6	45
10.6	35
13.0	36
8.4	
10.3±1.5(5) P1	44.0±10.0(4) P2

Notes:

1. Final values given as $\bar{x} \pm$ SD' (N).
2. P-values were derived using Students' t-test with Bessel's correction. Experimental values were compared to control data.

   P1 = $0.05 > P > 0.025$; P2 = $0.005 > P > 0.001$

cortico-striatal inhibitory neurones explain the differing involvement of cortical and striatal 5HT systems?

To investigate further the apparent reduction in cortical 5HT turnover, L-tryptophan was measured, using the method of Hess and Udenfriend (242, Methods C,IV), in a few animals to investigate the possibility of reduced substrate availability being a cause of the decrease of 5HT turnover. It was thought that minor changes in brain tryptophan concentrations might cause large changes in the rates of 5HT synthesis in brain (519), and therefore the possibility of changes in substrate levels could not be overlooked. L-tryptophan concentrations were therefore measured in the occipital cortex of a number of rats whose frontal cortices were simultaneously sampled and used for 5HIAA estimation, as recorded in Table A18 (Table A21). L-tryptophan concentrations were slightly reduced at 8 days post-implant, that is at a stage when cortical 5HIAA concentrations were unaffected, but at day 30, when cortical 5HIAA was low, L-tryptophan in the occipital cortex appeared to be considerably higher than normal. The significance of the latter datum is not clear (it is discussed further in the following section) but it does at least suggest that any apparent reduction in cortical 5HT turnover in this model cannot be explained in terms of reduced substrate concentrations. These observations could be criticised on the grounds that levels of tryptophan in occipital cortex may not reflect conditions in frontal cortex; this seems unlikely as Eccleston *et al.* (605) have found that the tryptophan concentrations in most areas of brain are roughly similar.

To provide some information of involvement of the serotonergic neurones at cell-body level, tryptophan hydroxylase activity was

Table A22

Tryptophan hydroxylase activity (n.mol. 5-HT equivalents produced/g brain/h) in pooled ipsi- and contralateral midbrain sections from cobalt-implanted rats.

A) Control values:
('Sham-operated' rats)

4.2
5.0
4.5
4.5 ± 0.3

B) Cobalt-implanted rats.

Day 6	Day 18	Day 20
5.4	4.4	3.6
4.5	4.1	3.8
4.7	3.8	4.0
4.9 ± 0.4	4.5	3.8 ± 0.2 (P1)
	4.2 ± 0.3	

Day 30	Day 75
4.0	5.2
3.9	5.0
4.0	4.2
4.1	
4.0±0.1	4.8±0.4

Notes:

1. Final values given as $\bar{x} \pm SD'$ (N).
2. P-values refer to comparisons between control and experimental data. Student's t-test with Bessel's correction was used. P1 = $0.05 > P > 0.025$.

Table A23

Tryptophan hydroxylase activity (n.mol. 5HT equivalents produced/g brain/h) in mid-brain sections of cobalt-implanted rats

A. Control animals ('sham operated')	Left	Right
1)	5.2	5.0
2)	5.0	5.0
3)	6.4	6.4
4)	3.2	3.4
5)	6.4	5.4
	5.2 ± 1.2	5.0 ± 1.0

B. Cobalt-implanted animals (25 days post-implantation)	Ipsilateral	Contralateral
6)	3.2	3.2
7)	4.0	2.0
8)	3.0	2.4
9)	3.4	2.2
10)	2.6	3.4
11)	2.6	3.4
12)	3.2	2.2
13)	3.2	3.2
	3.2 ± 0.4(P1)	2.8 ± 0.6 (P1)

Notes:

1. P-values refer to a comparison between control and experimental data, made using Student's t-test with Bessel's correction. P1 = $P < 0.001$.
2. Final values given as $\bar{x} \pm$ SD'.
3. All samples described here were processed together to minimise experimental error. The difference in base-line activity from values reported in table A22 may be due to the different sample of tetrahydrobiopterin co-factor used in the two experiments. This shaould not affect the internal consistency of these two experiments.

assayed in sections of mid-brain containing the anterior raphe nuclei (details of dissection, Methods A,IV; the assay used was based on that of Baumgarten et al. (243), Methods B,II).

In the first experiment (Table A22), mid-brain tryptophan hydroxylase activity appeared to show a reduction of approximately 10% between days 20 and 30 post-implant. Due to the small numbers of animals available, it was difficult to be sure whether the fall was significant so the experiment was repeated using only rats at 25 days post-implant. (Table A23). This second experiment seemed to indicate that a real reduction in enzyme activity was developing during the 3rd to 4th week post-implant, of around 35%. This last experiment had also been designed to investigate whether the reduced enzyme activity might be attributable to the cobalt that had been found to be present in the raphe (Table A13). One might, for example, hypothesise that cobalt in the cortex be taken up selectively into serotonergic axons and nerve terminals and then transported within the axons to the cell bodies in the raphe nuclei, with a subsequent effect either on the raphe cells generally or possibly on tryptophan hydroxylase specifically. There is evidence that cobalt is absorbed or taken up selectively by certain cells in the cortex (295), so the possibility could not be ruled out without investigation.

Tyrosine hydroxylase and tryptophan hydroxylase had both been tested for their sensitivity to cobalt (Table A11). As the two pterin-dependent hydroxylases behaved similarly in this respect, both enzymes were assayed in the cell bodies of the monoaminergic systems that projected to the cortex. The locus coereleus projects unilaterally to the fore-brain, and therefore if retrograde axonal transport of cobalt had taken place in noradrenergic neurones,

sufficient to contribute to any deterioration in the metabolism of the cells involved then one would expect a greater involvement on the side of the lesion. Mid-brain sections containing the loci were bisected, and each side assayed for tyrosine hydroxylase. (Table A9). All results were negative, in the sense that no changes in the enzyme activity could be detected, unilaterally or bilaterally. The raphe system on the other hand projects bilaterally, and one would not therefore expect any unilateral involvement in such a system, as measured by comparing tryptophan hydroxylase activity in ipsi- and contra-lateral raphe sections. These assays were performed using the same tissue samples as were used for the tyrosine hydroxylase assays, and in this case also no difference could be found between the ipsi- and contra-lateral sections (Table A23) and sections from control animals.

The atomic absorptiometric assay of cobalt in the brain showed that at 21 days post-implant, levels of cobalt in the raphe were around 3 and 5μg/g wet weight of tissue in contra- and ipsilateral raphe sections respectively (Table A13). This represents a cobalt concentrations of 45-75μM in the raphe section, assuming even distribution. Both tyrosine and tryptophan hydroxylase were unaffected by 10μM Co^{++}, in the conditions of the <u>in vitro</u> enzyme assay used, and showed a slight (around 10%) inhibition at concentrations of 100μM Co^{++} (Table A11). If the 3-5 μg cobalt per g tissue in the raphe sections was not evenly distributed, then a local concentration of cobalt of only 3 or 4 fold might have been sufficient to inhibit the activity of the pteridine-dependent hydroxylases. However the fact that tyrosine hydroxylase activity in the mid-brain sections

of cobalt-implanted rats did not differ from control values means that one either has to postulate that the cobalt in the raphe is associated primarily with the serotonergic cells, or look for a different explanation of the reduction in tryptophan hydroxylase activity.

INVESTIGATIONS INTO THE MODE OF THE APPARENT REDUCTION OF SEROTONERGIC TONE

These previous studies, showing a decrease in cortical 5HIAA concentrations and in tryptophan hydroxylase activity in the raphe nuclei, together with unchanged concentrations and rates of uptake of 5HT in the cortex, seemed to suggest that the apparent reduction of 5HT turnover in the cortex may have been due to a response of the serotonergic system as a whole, rather than simply a local, cortical response to the cobalt implant. It was hoped that one might obtain information concerning the maximal turnover capacity of the serotonergic system by giving experimental and control animals loading doses of L-tryptophan (180, 236), and then measuring concentrations of brain 5HT and 5HIAA at set times after the administration of the L-tryptophan. If the apparent reduction of 5HT turnover already noted reflected a pathological involvement of the 5HT system, then presumably the maximal turnover capacity would be lowered in proportion to the apparent reduction; if the suppression was functional, then the stimulus of a tryptophan load would result in a proportionately smaller difference between cobalt and control rats, or even no difference at all. Ideally one would have wanted to measure tryptophan hydroxylase activity in the affected cortical areas, but unfortunately the method used was insufficiently sensitive.

The experiment was designed as follows: a large group of the experimental animals was divided into two smaller groups, one of

Table A24

5-hydroxyindolacetic acid (ng/g wet weight of tissue) in frontal and occipital cortex of control and cobalt-implanted rats in the absence of, or subsequent to, tryptophan loading.

A) Control animals
(glass-implanted)

Unloaded		Tryptophan loaded	
Frontal	Occipital	Frontal	Occipital
197	246	434	351
268	276	509	396
219	197	482	316
		206	180
228 ± 29(3)	239 ± 32(3)	407 ± 119(4)	310 ± 80(4)

B) Cobalt-implanted rats, lesion cortex

Unloaded		Tryptophan loaded	
Frontal	Occipital	Frontal	Occipital
88	57	447	276
113	79	303	294
61	154	224	294
184	149	276	263
		408	263
111 ± 45 (4)	109 ± 42(4)	331 ± 83(5)	278 ± 13(5)

C) Cobalt-implanted rats, contralateral cortex

Unloaded		Tryptophan loaded	
Frontal	Occipital	Frontal	Occipital
79	96	337	228
96	44	320	241
88	176	289	311
263	224	351	272
		167	228
131 ± 76(4)	135 ± 69(4)	300 ± 73(5)	256 ± 31(5)

Notes:

1. Final values given as $\bar{x} \pm SD'$ (N).
2. Using Student's t-test with Bessel's correction, it was shown that cortical 5HIAA in unloaded, cobalt-implanted rats was significantly lower ($P < 0.005$) than control cortical values.
3. Tryptophan-loaded animals were given 400 mg/kg L-tryptophan i.p., 2h before killing.
4. Assay method no. 1 was used (Methods CV).

Table A25

5-hydroxytryptamine (ng/g wet weight of tissue) in frontal and occipital cortices of cobalt-implanted and control rats in the absence or subsequent to L-tryptophan loading

A) Control animals (glass-implanted)

Unloaded		Tryptophan loaded	
Frontal	Occipital	Frontal	Occipital
519	519	667	741
615	426	833	630
370	259	870	611
		685	714
501 ± 83(3)	401 ± 107(3)	763 ± 88(4)	674 ± 69(4)

B) Cobalt-implanted rats, ipsilateral cortical tissue (25 days post-implantation)

Unloaded		Tryptophan loaded	
Frontal	Occipital	Frontal	Occipital
481	426	1560	963
426	389	630	593
472	324	685	593
583	343	1040	815
		1200	611
490 ± 57(4)	370 ± 39(4)	1023 ± 343(5)	715 ± 149(5)

C) Cobalt-implanted rats, contralateral cortical tissue

Unloaded		Tryptophan loaded	
Frontal	Occipital	Frontal	Occipital
444	389	759	741
352	333	704	509
463	361	796	815
463	380	944	788
		741	788
431 ± 40(4)	366 ± 19(4)	788 ± 83(5)	724 ± 110(5)

Notes:

1. Final values given as $\bar{x} \pm SD'$ (N).
2. Student's t-test with Bessel's correction was used to compare experimental with control data. In no case were 5HT values in the experimental animals significantly different ($P > 0.2$) from the relevant control values.
3. Tryptophan-loaded animals were given 400 mg L-tryptophan/kg, i.p., 2h before killing.
4. Assay method no. 1 used (Methods CV).

these sub-groups being given cobalt implants and the other glass implants. At 25 days post-implant, when the reduction in cortical 5HIAA levels and mid-brain tryptophan hydroxylase activity had been found to be ongoing (Tables A17,18,22,23), each of these sub-groups was again divided in two, and one half was given a loading dose of L-tryptophan (Methods, D,IV). Two hours after the L-tryptophan administration, all animals were killed, and brains were stored in liquid nitrogen for subsequent assay.

The method of L-tryptophan administration and the dosage used, were chosen to create conditions resulting in a plateauing of brain 5HT and 5HIAA concentrations at 2h after injection, using the smallest possible dose to achieve this (180,236). In retrospect, this may have been a wrong decision - it may have been better to use smaller doses of L-tryptophan, because, with the dose used (400 mg L-tryptophan/kg), minor differences in rates of 5HT turnover might well have been swamped.

On comparison of the unloaded groups, the cobalt-implanted rats again demonstrated reduced concentrations of 5HIAA and normal 5HT concentrations (as measured in cortical tissue) in comparison to the control values (Tables A24,A25). This was in agreement with the results already quoted (Tables A16-19). A comparison of the data from the L-tryptophan loaded rats shows that, when stimulated by a large tryptophan load, the serotonergic system in the cobalt-implanted rat was apparently capable of increasing its turnover to the same extent as in the control rat. This does not at first sight appear to be reconcilable with the reduction in mid-brain tryptophan hydroxylase activity found in this model (Tables A22,A23). However, even if rates of 5HT synthesis are depressed in the cobalt-implanted rat, this might not be uncovered by the tryptophan loading test for the following reasons. Studies by Eccleston *et al.* (236) have found

that levels of 5HT in brain plateau at 1 hour after tryptophan administration in the control rat. Even a reduced level of tryptophan hydroxylase activity might, in conjunction with the reduced MAO activity found in this model (Table A4), achieve cerebral 5HT concentrations at 2 hours after tryptophan administration similar to those found in the control animals. Furthermore, a block of metabolite transport as has been postulated in this model would, by elevating 5HIAA levels, also contribute to concealing the effects of a reduction of tryptophan hydroxylating capacity.

In this context, how can one reconcile the findings of raised (occipital) cortical tryptophan at day 30 post-implant, and an apparent reduction in frontal cortical 5HT turnover, with the fact that a tryptophan loading dose can increase 5HT turnover in the same model?

There are various reports in the literature of tryptophan levels in brain being altered by drugs that affect 5HT levels and/or rates of synthesis. For example, reserpine, d-amphetamine, lithium salts, and other treatments such as electroconvulsive shock and heat stress, have all been reported to raise the rate of 5HT turnover in mammalian brain tissue (296,285,297-302). All of these treatments have been shown to increase brain levels of tryptophan concurrently (300, 301, 302). Conversely, para-chloramphetamine (pCA) causes a reduction in brain 5HT, and lowers brain tryptophan also (302).

More recently Knapp and Mandell (267) have demonstrated that, after lithium administration in the rat, the high-affinity uptake of tryptophan into 5HT nerve endings in cortex increased in capacity (i.e. the Vmax. value increases) together with a correspondingly increased turnover of 5HT in the nerve-endings. 5HT synthesis at

first increased in step with the increased substrate levels, but after a series of adjustments in the cell bodies involving a reduction of the amount of tryptophan hydroxylase formation, 5HT turnover returned to normal, while the uptake mechanism was still stimulated. If at any time during this series of adjustments 5HT turnover fails to keep in step with tryptophan uptake, concentrations of tryptophan in the brain might well be affected, depending on the amount of the amino acid that could be metabolised via other pathways.

This may explain the findings in the cobalt rat. It is possible, for example, that the cobalt ion stimulates the uptake mechanism for tryptophan in a way similar to lithium (267) and caesium (300), and this effect, together with a reduction of 5HT turnover would probably result in raised brain tryptophan levels. It must be added, however, that Van Gelder and Curtois (533) examined amino acid levels in the primary and secondary foci in the cobalt-implanted cat, but found no change in brain tryptophan. Colasanti and Craig (454) have also reported alterations of cerebral 5HT turnover in a cobalt-treated rat. It must be stated, however, that their model is probably fairly unlike the model reported on in this thesis; they report applying 30mg of cobalt powder directly onto the surface of the parietal cortex, approximately 50-60 times the amount of cobalt used in our model, and almost certainly producing gross lesions not only in the cortex but also all sub-cortical structures. They examined 5HT turnover in whole brain, 6 days after cobalt administration, by measuring the rate of increase in cerebral 5HT levels after MAO inhibition, and measuring the rate of decline in 5HIAA in

parallel. Both of these parameters indicated that 5HT turnover was significantly low in the cobalt-treated animal. They could not find any evidence for similar involvement of NAd or DA systems, and also reported finding no alterations in whole brain NAd, DA and 5HT levels. Korf et al. (581) have reported a pattern of changes in serotonergic metabolism after probenecid which appears to resemble in some ways some of the data presented in this thesis. Using probenecid to block metabolite transfer in the rat, they found that in this case brain tryptophan levels and 5HT turnover appeared to be uncoupled; total serum tryptophan fell, and brain tryptophan nearly doubled, yet 5HT turnover appeared to remain unchanged. They suggested that the probenecid inhibited the binding of tryptophan to serum albumen, and that the resultant increase in plasma free tryptophan provided for the rise in brain tryptophan, and also suggested that, at least in certain cases, brain 5HT turnover may not always be linked to brain tryptophan. If we postulate that rates of tryptophan hydroxylation are proportional to tryptophan levels inside the serotonergic neurones, and that this intraneuronal pool of tryptophan, although usually representative of total brain tryptophan concentrations, may in certain circumstances increase or decrease in size relative to the total brain tryptophan pool, we then have a model where intraneuronal rates of tryptophan hydroxylation may appear to become uncoupled from brain tryptophan concentrations. It is possible, for example, that this model might explain the fact that in the cobalt-implanted rat, an increase in cortical tryptophan at day 30 occurs together with an apparent reduction of cortical 5HT turnover (Tables A17,18,21). The increase in tryptophan levels, after all, could include tryptophan non-specifically bound to protein, and unavailable to the hydroxylase.

INTERACTIONS BETWEEN SEROTONERGIC TONE AND THE SEIZURE THRESHOLD

The work that is relevant to this area falls into three basic categories:

I The effects on the seizure threshold, of treatments that affect the serotonergic system. This category includes pharmacological and surgical techniques and also certain metabolic errors of monoamine metabolism.

II Alterations of serotonin metabolism in various epilepsy models.

III The effects of known anticonvulsant and convulsant drugs on 5HT metabolism.

SECTION I. THE EFFECTS ON THE SEIZURE THRESHOLD, OF TREATMENTS THAT AFFECT THE SEROTONERGIC SYSTEMS

The mechanical or electrical lesioning of the raphe nuclei or ascending tracts by Jouvet, Pujol and others has yielded some interesting data: However, there is a certain amount of controversy surrounding this work, as the raphe nuclei are so diffuse that any comprehensive lesioning almost inevitably involves the destruction of other tracts (487). In addition, most of this work has been directed towards an analysis of sleep rather than of epilepsy.

The use of drugs that alter 5HT metabolism is of more interest. This can be divided into two groups of data, one concerning the effects of an increased serotonergic tone and the other documenting the effects of a lowered tone.

I(a) Drugs that lower effective central 5HT levels by receptor blockade

Certain drugs thought to act as central 5HT receptor blockers have been investigated in certain epilepsy models. The drugs

generally used are lysergic acid diethylamide (LSD) and 2-bromo-d-lysergic acid (BOL). LSD has been found to completely block the behavioural and EEG signs of epilepsy in the photosensitive baboon (127, 128, 112). However there is evidence that LSD (and BOL) only block the excitatory action of 5HT (129, 178, 482), and if, as the microiontophoretic studies imply, cortical 5HT is primarily an inhibitory neurotransmitter (552), then one is led to suspect that these drugs' anticonvulsant action may not be mediated via the serotonergic system.

Meldrum et al. (127), screening various hallucinogens and 5HT receptor blockers in the photosensitive baboon, discovered that the anticonvulsant action of LSD and psylocybin could have been mediated in part by the effect both drugs had on inhibiting transmission in the afferent visual pathway. Methysergide, also an anticonvulsant in this model, did not have this effect, and they concluded that the anticonvulsant effect of these drugs was therefore primarily mediated via the serotonergic system.

It is not completely clear whether LSD, BOL and methysergide act as 5HT antagonists or whether they are partial agonists (129, 178, 482) at serotonergic receptors. Furthermore, LSD has been found to have a dopamine-receptor stimulating action (324, 325), so it becomes rather difficult to specify exactly what the mode of action of these anticonvulsants is. One also cannot rule out the possibility that the effects on the cortex of these drugs may be secondary to their effects on sub-cortical structures. LSD has been reported to inhibit cells in the raphe (127, 128, 259, 260), and altering raphe tone on the cortex may have an effect on a cortical epileptic focus quite different to that of a 5HT receptor antagonist or agonist, as

the cortical distribution of such a drug may not parallel the map of the cortical serotonergic innervation.

Of this group of drugs, methysergide has been used clinically. At least three clinical trials have been reported (135, 136, 137), all finding that methysergide was effective in treating certain cases of temporal lobe and other epilepsies.

I(b) Drugs that lower central 5HT levels by inhibiting 5HT synthesis

The two drugs most commonly used to reduce central 5HT levels are para-chloramphetamine (PCA) and para-chlorphenylalanine (PCPA). Only PCPA has been used in the study of epilepsy to date. An amino-acid analogue, its irreversible inhibition of tryptophan hydroxylase (488) can be prevented by pre-treatment with a protein synthesis inhibitor (490).

PCPA lowers the seizure threshold in the mouse and rat electro-shock and pentylenetetrazole convulsion models (45, 46, 134) and the photosensitive baboon (113). However there are other reports that PCPA elevates the seizure threshold in the audiosensitive mouse (139, 565), suggesting that the neuronal substrate in the mouse electroshock and audioshock models may be different.

Unfortunately the biochemistry is not absolutely clear, as PCPA also inhibits tyrosine hydroxylase, though not to the same extent as tryptophan hydroxylase (491, 492, 493). This means that here again one cannot be completely certain about the relationship between serotonergic tone and the seizure threshold.

I(c) Drugs that increase central serotonergic tone

Various workers have investigated the effects of directly administered 5-hydroxytryptophan (5HTP) or 5-hydroxytryptamine (5HT) on the seizure threshold.

The intracranial administration of 5HT in mice into the frontal lobe has been reported to afford some degree of protection against audiogenic and pentylenetetrazole seizures (59, 130). In rats, repeated intracranial doses of 5HT have been reported to antagonise the convulsant effects of pentylenetetrazole and picrotoxin (150), although single intracranial doses were ineffective (150, 151, 430). In an analogous fashion, the short-term 5HT-releasing action of p-chloramphetamine has also been shown to protect mice from audioshock. (130, 131). There is also one reference (431) stating that the systemic administration of 5HT has a clinically useful anticonvulsant effect. This is difficult to evaluate in the light of evidence that 5HT does not pass the blood-brain barrier in significant amounts (179), but possibly the anticonvulsant effect in this case is mediated by a peripheral action of 5HT on sensory afferents. This possibility is examined in the final discussion section.

As 5HTP is able to pass the blood-brain barrier, it can be given systemically. Large doses of 5HTP have been found to raise central 5HT concentrations and the seizure threshold in parallel in reserpinised mice (44,53). 5HTP administration has also been reported to partly protect the methionine sulphoximine pre-treated rat from audiogenic seizures (132), to depress EEG responses to photic stimulation in the photosensitive baboon (40), and to depress the epileptiform after-discharge in the chronically isolated cortical preparation in the cat (5). Interestingly enough, this last effect could be blocked by methysergide.

Pre-treatment with MAO inhibitors has been reported to enhance the anticonvulsant effect of 5HTP in the photosensitive baboon (112) and

audiosensitive mouse (139). However there are also available reports of 5HTP inducing EEG abnormalities in the photosensitive baboon (113), and precipitating epileptic behaviour in the "alumina cream" focus in the cat and monkey (50).

Tryptophan administration, with or without an MAO inhibitor, has also been used to raise central 5HT levels. The combination of tryptophan plus an MAO inhibitor has been shown to raise the seizure threshold in the rat electroshock model (6, 47), and there are several reports of 5HTP, tryptophan alone, and tryptophan plus an MAO inhibitor all being clinically effective in the treatment of myoclonus and various other types of epilepsy (71, 420, 461, 462, 463). There is some evidence that these forms of anticonvulsant therapy are most effective with those patients who present with low levels of 5HIAA in the lumbar CSF (420).

However there is at least one conflicting report (51), that found that tryptophan actually activates epileptic foci in some patients.

The contradictory results described above, some studies indicating that an increase in central 5HT may be anticonvulsant in some instances, but may precipitate EEG abnormalities in others, indicate that it would be wrong to assume a first order relationship between central 5HT concentrations and the seizure threshold. This point is very effectively demonstrated by Kellog et al. (139), who found that the seizure threshold in audiosensitive mice was raised both by p-chlorophenylalanine, an inhibitor of 5HT synthesis and by 5HTP, given in conjunction with an MAO inhibitor. There are several possible explanations for this. First, one can postulate model and/or species differences to reconcile some of the data. Second, one

could hypothesise that there may be an optimal range of central 5HT concentrations above or below which cortical malfunction may develop. Although cortical 5HT is thought to be mainly inhibitory (552), when present in excess it might also act as an excitatory neurotransmitter, possibly at physiologically inappropriate receptor sites if overspill occurs from normal storage sites, or when, for example, 5HTP is decarboxylated by the unspecific aromatic aminoacid decarboxylase (AAD) in non-serotonergic neurones.

Third, one can investigate the possibility that the apparently contradictory results are due to the non-specificity of the drugs used to manipulate the serotonergic system. For example, the side-effects of LSD and PCPA have already been mentioned. Even 5HTP, the immediate precursor of 5HT, may act on central systems other than the serotonergic one. It has been suggested that, as 5HTP and Dopa are probably metabolised by the same decarboxylase (AAD) to form 5HT and DA respectively, large doses of either substrate may, by swamping the enzyme's decarboxylating capacity, inhibit formation of the other amine (425). Until very recently, the MAO inhibitors available have not been sufficiently specific to differentiate between the 'A' and 'B' forms of MAO (606), so in those experiments where MAO inhibitors were used one cannot completely disciminate between the possible effects on any of the monoaminergic systems. There is also available evidence that various types of neurone in the CNS work intimately together, with effects on one system being followed by compensatory changes in another. For example, an increase in cortical 5HT turnover has been reported after sectioning the noradrenergic dorsal bundle (471), and conversely an increase in central 5HT concentrations (after

tryptophan administration) has been shown to be correlated with a reduction of the threshold to acetylcholine-induced EEG seizure discharges (49). Furthermore, it now seems likely that drugs thought to act specifically on α- and β-adrenoreceptors, and also histamine receptors, may, by affecting blood-flow in certain brain areas, alter local pO_2, pCO_2 and pH values (437). These factors have been shown to alter gabaminergic and serotonergic tone (161, 163), and thus again we have a situation where an apparently specific drug may indirectly affect a range of neuronal systems.

SECTION II ALTERATIONS OF 5HT METABOLISM

Alterations of 5HT metabolism in models of epilepsy

Much of this work has been done on the audiosensitive mouse model. An early paper by Schlessinger *et al.* (95), detailed the relationship between age, brain amine levels, and seizure susceptibility in the audiosensitive mouse and reported an inverse correlation between seizure threshold and levels of central NAd and 5HT. More recently the work from Schlessinger's group has concentrated on the serotonergic system. Brain 5HT levels have been shown to follow circadian rhythms, being greatest at noon and lowest at 20.0 to 24.0 hrs (308, 309). Subsequently 5HT synthesis and turnover were shown to follow the same pattern (310). Finally a study by Schlessinger and Schreiber (52) established that the diurnal rhythm of the seizure threshold paralleled the rhythm of the serotonergic system, being at its lowest at night and highest during the day. No apparent involvement of NAd could be established on this study.

Another model, the audiosensitive rabbit, has also been reported to display significantly lowered brain-stem 5HT levels during the

period of maximal audiosensitivity (455), but at this date the model has not been investigated further.

Various workers have looked at aspects of 5HT metabolism in other models; there is one report of increases in brain 5HIAA during seizures in the tungstic acid model (10), and another detailing findings of raised brain concentrations of 5HT in rats immediately after electroconvulsive treatment (428). Although there is obviously no directly analogous clinical information, concentrations of 5HIAA have been measured in the CSF of patients given ECT for depressive illness (142), but in this case no significant changes were detected after a course of treatment.

Reports of altered 5HT metabolism in cases of human epilepsy

There has been a lot of work done in this area in the last decade. Many of the data are contradictory. This is probably due in part to the anticonvulsant drugs which many patients were receiving, which have themselves an action on 5HT metabolism. Various aspects of methodology are also suspect. Clinical research is hampered by the limitations of sampling. Much of the work reported on in this section on clinical studies is based solely on assaying for 5HIAA in lumbar CSF samples. However, there is evidence that suggests that this technique may be of little value in monitoring central 5HT turnover. It should be noted that any theory attempting to link CSF concentrations of 5HIAA in a simple, first-order relationship with the effectiveness of the serotonergic system, or with a biochemical pattern of epilepsy, does not take into account the fact that there are thought to be two pools of 5HIAA contributing to CSF 5HIAA levels (80, 81, 82). One pool represents 5HT that is released from serotonergic nerve terminals, is effective at post-synaptic sites and

catabolised extraneuronally. The other pool is derived from 5HT that is synthesised in serotonergic neurones in excess to requirements and storage capacity, and is a substrate for intraneuronal MAO. Only the contribution of the first pool to CSF 5HIAA concentrations represents the degree of serotonergic tone; overall rates of 5HT turnover as measured by assaying CSF 5HIAA do not therefore accurately represent the activity of the central serotonergic system. The importance of this concept is that MAO in serotonergic neurones can thus uncouple serotonergic tone from brain tryptophan levels, and ultimately dietary tryptophan, thereby strengthening the independence of neuronal function.

Secondly, it has been shown that, in various experimental animals, concentrations of 5HIAA in lumbar CSF reflect changes in spinal cord 5HIAA, rather than concentrations of 5HIAA in cisternal CSF (258, 590-592). This may also be the case in the clinical situation; and furthermore, the areas of fore-brain involved in epileptic phenomena are innervated by the anteriorly-projecting rostral raphe nuclei; the descending serotonergic systems, deriving from the caudal raphe nuclei, may not always be affected in proportion to the ascending serotonergic systems. It would be a mistake to assume that a biochemical homogeneity (the neurotransmitter both systems have in common) denotes a common physiological response. Various workers have attempted to solve some of these problems by taking samples of mixed CSF, and it has also recently been suggested (255, 256) that a parallel estimation of tryptophan with the 5HIAA in CSF samples might provide more information, as to the central 5HT turnover.

If CSF tryptophan correlates with brain tryptophan levels, then such a dual analysis could determine whether the reported decrease in 5HT turnover during depression or epilepsy is contributed to by changes in substrate availability in the brain, or an effect on the rate-limiting hydroxylation step. Although correlations have been found between blood and lumbar CSF tryptophan levels in patients receiving tryptophan orally, (256), no such relationship between CSF tryptophan and CSF 5HIAA has so far been found (257, 258). If the rate of central 5HT turnover is directly related to substrate concentrations, as some authors think (180, 188, 189, 190), and if CSF tryptophan is linked to blood and/or brain tryptophan, then one would expect CSF tryptophan and 5HIAA concentrations to be related. In fact the only study reporting such a correlation was basdd on an experiment using genetically standard animals in a controlled environment (255). The value of such dual assays is therefore not yet ascertained.

CLINICAL DATA

One study (8) found significantly reduced 5HIAA (60% of control values) in the lumbar and mixed CSF of temporal lobe and Grand Mal epileptics. Levels of homovanillic acid (HVA) in the CSF were, in this study, reported as normal. Two corroborative studies (9, 87) indicated that, after the inhibition of CSF metabolite transport (using probenecid), the accumulation of 5HIAA in the CSF of otherwise unmedicated epilepsy patients was significantly lower than control values. A third study (36) by the same authors found a 50 per cent decrease in lumbar and mixed CSF 5HIAA levels in epileptic children; however, in this trial they also found a similar decrease in CSF HVA levels, in contrast to their earlier findings. The authors

speculated that 5HT turnover in their patients might have been even lower than these findings suggest, as most of the patients were being treated with diphenylhydantoin, a drug which has been reported both to increase 5HT turnover in brain (36, 426, 427, 428) and to inhibit 5HIAA transport (36). There have been surveys by other workers (32, 33, 34) also detailing findings of lowered 5HIAA and HVA in the CSF of epilepsy patients.

It seemed relevant at this point to include in this section a discussion of the effects of anticonvulsants on 5HT metabolism, as so many of the reports on CSF metabolite levels are based on samples from patients who are receiving medication of some sort.

SECTION III EFFECTS OF ANTICONVULSANTS ON 5HT METABOLISM

Already, before 5HT had been established as a central neurotransmitter, a fairly exhaustive survey had been carried out by Bonnycastle et al. (30) concerning the effects of various anticonvulsants on brain 5HT concentrations in the rat. Phenytoin, methoin, troxidone, paramethadione, phensuximide, phenacemide, primidone, phenobarbitone and sodium bromide were all reported to cause significant increases in brain 5HT; and it was suggested that the drugs exerted their anticonvulsant effect primarily by elevating serotonin concentrations. However it was shortly afterwards established that lower but still anticonvulsant (i.e. protective against electroshock) doses of diphenylhydantoin did not alter rat brain 5HT (548), nor did meprobamate, another anticonvulsant drug (547). Two non-anticonvulsant barbiturates pentobarbitone and hexobarbitone, also raised brain 5HT concentrations (547), and there has been at least one report (428) of a convulsant, pentylenetetrazole, raising rat brain 5HT, so the relationship between anticonvulsant efficacy and an effect on 5HT levels became rather blurred.

More recently an extensive study by Chadwick et al. (29) attempted to explain the findings of altered 5HIAA concentrations in CSF from epileptics as a result of their medication, as well as of the underlying pathology. They analysed the CSF of patients suffering from various forms of epilepsy and found that in CSF from untreated patients 5HIAA and HVA values were close to normal. Those patients who were being treated with anti-convulsants, notably diphenylhydantoin and phenobarbitone, appeared to have raised levels of these metabolites. They also found that those subjects who had been receiving subtherapeutic doses of the drugs did not have significantly raised CSF values, and conversely those patients manifesting drug toxicity, with very high blood levels of the drugs, had the highest CSF metabolite levels. They did not, unfortunately, present any data concerning the effects of diphenylhydantoin and phenobarbitone on the concentration of 5HIAA and HVA in the CSF of non-epileptic control patients.

Although other workers have been unable to show this correlation between plasma levels of diphenylhydantoin and CSF concentrations of 5HIAA and HVA in epileptic children (9,87), Chadwick's paper seems to show that the reduced CSF concentrations of 5HIAA and HVA reported in other clinical studies (8,9,32-34,36) could not have been a direct result of their medication, as some critics had suggested. It also suggested that the anticonvulsant drugs acted primarily on central 5HT and DA systems, although it is unclear whether the main action is on amine turnover, release, uptake or transport mechanisms.

One group of workers (36) has found that diphenylhydantoin and phenobarbitone slow down the rate of removal of 5HIAA from the CSF in the rat, and reduce the rate of 5HT and NAd oxidation in brain slices.

This first action would tend to raise CSF 5HIAA concentrations, the second would have the reverse effect. If one or other action predominates at different doses of the drug, this could explain some of the conflicting results on 5HIAA levels in the CSF of epileptic patients receiving anticonvulsant medication. The effect on dopaminergic systems may be similar, as phenobarbitone has been shown to reduce DA turnover in rat brain (37,61).

The action of diphenylhydantoin and phenobarbitone on functionally reducing 5HT catabolism would explain the previous findings of these two anticonvulsants raising 5HT concentrations in rat brain (30, 31, 152, 153).

There is available further evidence linking the anticonvulsant effect of diphenylhydantoin to its action on the metabolism of 5HT; the rate of 5HT increase in the brains of rats given diphenylhydantoin has been found to parallel very closely the development of the anticonvulsant's effect on the seizure threshold to both electroshock (155) and to hexafluorodiethyl-induced seizures (156). These findings have, however, been disputed by other authors (157, 158, 159). It must be clearly stated that none of these pieces of evidence can establish whether the apparent changes in serotonergic metabolism are fundamental to the anticonvulsant effect or merely reflect a cortical inhibition, which itself may not be primarily serotonergic in origin.

The theory of a primarily serotonergically mediated anticonvulsant effect has had its supporters and detractors. Perhaps one of the best studies in support of the hypothesis was one by Meyer and Frey (41), who examined the effects of altered brain amine levels on the ability of phenobarbitone to protect against electroshock and

pentylenetetrazole convulsions. Depleting central 5HT and NAd by pre-treatment with p-chlorophenylalamine and α-methyltyrosine rendered the anticonvulsant phenobarbitone ineffective. Cyproheptadine and phentolamine, 5HT and NAd receptor blockers respectively had the same effect, while 5HTP and L-Dopa administration augmented the anticonvulsant action of phenobarbitone.

Other authors contested these findings. Rudzik and Mennear (140, 141) used α-methyltyrosine, α-methyldopa and α-methyltyrosine to lower central NAd levels, but found them all ineffective in antagonising diphenylhydantoin. They suggested that diphenylhydantoin therefore may exert a part of its anticonvulsant action through mechanisms other than alteration of monoamine metabolism, although they only examined one model, the audiosensitive mouse.

Bonnycastle et al. (429) cite evidence that pretreatment with diphenylhydantoin can augment the increase in brain 5HT after 5HTP administration, and can also reduct the extent of 5HT depletion in rat brain following reserpine. Green and Graham-Smith (80,81) have published some very interesting data concerning the effects of diphenylhydantoin on 5HT turnover; after a dose of diphenylhydantoin that had previously been reported to enhance the hyperactivity in the rat after tryptophan loading and a MAO inhibitor (82), they established that the same dose on its own did not increase 5HT levels, nor did it alter synthesis rates, 5HT uptake, or post-synaptic response to 5HT agonists. They suggested that the drug's action at that particular dose may have been to increase the amount of 5HT available for release at the nerve terminals, by effecting some redistribution of the neurotransmitter between different 5HT pools. They also looked at

the effects of repeated doses of diphenylhydantoin and found that after a short course of the drug the rate of 5HT synthesis in brain apparently doubled. There was no increase in 5HT levels, so that catabolism was keeping in step with synthesis; the 5HIAA concentration in the CSF increased twofold, agreeing with the earlier observation that diphenylhydantoin and phenobarbitone had the ability to raise CSF 5HIAA levels.

It is of interest to note here that lithium-treated rats display similar changes in 5HT synthesis, and the behavioural effects may be comparable too (83). There are also papers available (84, 85, 219) describing findings of a significant rise in concentrations of 5HIAA in the lumbar CSF of lithium-treated manic patients. The fact that lithium appears to act on 5HT metabolism in a manner resembling that of diphenylhydantoin, yet is not an anticonvulsant (86) tends to detract from the theory of anticonvulsants as primarily acting by altering serotonergic nerve function.

It may also be critical to know not only the type and amount of medication a patient is receiving, but also for how long that treatment has been going on. Goodwin et al. (262,263) have reported finding elevated 5HIAA levels in the CSF of patients receiving lithium for 5 days, and sub-normal levels of 5HIAA at 21 days. They postulated on adaptative mechanism, which tended, after the primary action of lithium, to return the neurophysiological balance to normal.

Poitou and Bohuon (347) have examined the action of Li^+ on catecholamine metabolism in the rat, and found a similar biphasic pattern here. After 5 days of Li^+ treatment, NAd synthesis had increased above normal, but was at normal or subnormal rates at 15 days; DA

synthesis was also reduced. They suggested that even if NAd synthesis was increased at day 5,this was associated with a reduction in effective NAd levels, because Li^+ stimulates intraneuronal catecholamine release, with a subsequent increase in the metabolites produced by deaminative oxidation by MAO. This is backed up by two earlier papess, a report (344) that Li^+ reduces the extra-neuronal release of NAd after electrical stimulation, and a clinical paper (348) recording findings of raised MAO metabolites of catecholamines in CSF after lithium therapy.

Knapp and Mandell (264, 265, 267) have suggested that a whole array of adaptive mechanisms exist whereby a neurone or neuronal system can absorb a stimulus; adaptations that affect the system in such a way that receptor stimulation always tends to return to normal levels. For example, after amphetamine administration in the rat, they find decreased tryptophan hydroxylase activity in the raphe after 30 minutes, and in the cortex after 60 minutes. The drug does not inhibit the enzyme <u>in vitro</u>, but it does increase receptor stimulation by increasing neurotransmitter release and blocking re-uptake (266). It was suggested that the rapid reduction of tryptophan hydroxylase activity was due to a compensatory mechanism called into play by the increased receptor stimulation, and consequent neuronal feedback. The mechanisms which may be involved include substrate uptake, the activity of the synthetic enzymes, and possibly other factors such as availability of reduced co-factor. In the case of the adaptive responses to Li^+, the paper by Knapp and Mandell (267) reports the following sequence; after 3 to 5 days of Li^+ administration, first the high affinity uptake mechanism for tryptophan in the striatum was increased; second, the

tryptophan was metabolised more rapidly to serotonin in a striatal synaptosome fraction; third, soluble tryptophan hydroxylase activity in the raphe decreased. After 21 days of lithium administration the synthetic rate in the synaptosomes dropped to normal, even when the high affinity uptake of tryptophan was still being stimulated by the Li^+. In other words, after a stimulation of substrate uptake and 5HT synthesis in the nerve terminals a compensatory decrease in synthetic enzyme occurred in the cell bodies, which was eventually mirrored in the nerve terminals.

In practical terms this meant that in the attempt to elucidate the involvement of the serotonergic circuitry in the cobalt rat, it would have been unsufficient to record any one parameter of the neurones' viability. Simply measuring raphe tryptophan hydroxylase would not necessarily have indicated how the cortical serotonergic connections were functioning, and similarly any assay of cortical 5HT would not have given sufficient indication of the possibility of an altered state of affairs in the cell bodies.

Metabolic Errors and the Seizure Threshold

There is one last category of work available concerning relationships between amine levels and seizure threshold, and this refers to studies on patients with inborn errors of metabolism, some of which affect levels of various neurotransmitters directly or indirectly. However, most of this data is rather confusing as in most cases the spectrum of effects on the overall metabolism is too wide to permit any one-to-one relationship to be picked out. The best-studied disorder is phenylketonuria. Approximately 50% of patients with this disease display epileptiform abnormalities, and overt epileptic

behaviour (268-271), including petit mal and grand mal. The primary lesion is an absolute or relative deficiency of phenylalanine hydroxylase. Phenylalanine is instead converted to phenylpyruvate and then to phenylacetate and phenyllactate.

The effects on the various neurotransmitters are as follows; first, the increased concentrations of phenylalanine in the blood inhibit the transport of other amino acids into the brain, including tryptophan (272, 273), with the result that central 5HT turnover is depressed in these patients, as indicated by the low CSF 5HIAA concentrations (275) they present with. Second, the synthesis of the catecholamine neurotransmitters NAd and DA is also affected by the deficiency in phenylalanine hydroxylase (276, 277).

Unfortunately the story does not end here; for the phenylalanine metabolites, phenyllactic and phenylacetic acid, which are present in abnormally high levels in the brains of phenylketonuric patients, are very potent inhibitors of glutamic acid decarboxylase (GAD) (278, 279), and the consequent reduction in GABA synthesis is also a probable factor in the low seizure threshold. If phenylketonuric patients are given a phenylalanine load, all the metabolic abnormalities mentioned above are exacerbated, and often fits and convulsive behaviour are precipitated (280). A phenylalanine deficient diet may return the amino acid and amine patterns to normal; for example a return to normal levels of free serum 5HT has been reported (274), but this is not always associated with behavioural or EEG improvement (281) as permanent damage may have already been done to the neural substrate.

To summarise the preceeding three sections, it can be stated broadly that CNS concentrations and turnover of 5HT, NAd and possibly DA are affected in various forms of epilepsy. Changes in the central availability of all three amines alter the seizure threshold; the effects of all three systems are inter-related, and there is reason to suggest that certain anticonvulsant drugs exert at least a part of their action by modifying the monoaminergic neuronal systems.

INTRODUCTION TO THE PHARMACOLOGY SECTION

From a thorough survey of clinical and experimental literature two broad and contradictory trends had emerged. First, the physiological increase in the activity of the serotonergic system occurring during non-REM sleep appears to facilitate the appearance and spread of discharge behaviour in the EEG (314, 315, 316, 317). Second, a completely non-physiological increase in brain levels of 5HT such as is caused by 5HTP loading (469, 44, 130, 131, 132, 40, 132), or the combination of tryptophan loading and an MAO inhibitor (6, 47, 7, 420), is very effective in reducing or suppressing epileptic behaviour in a variety of models. A third body of data refers to the effects of a less extreme, but still unphysiological, amplification of the serotonergic system as caused by tryptophan loading alone. Those workers who have examined the effect of this treatment on the seizure threshold have published conflicting data. Some reports suggest that tryptophan loading is effectively anticonvulsant, (461, 462), while others find that tryptophan loading actually activates existing epileptic foci (50, 51). It would have been possible to construct some bridging theory around the non-specificity of the drugs used to manipulate the serotonergic system, but this seemed unsatisfactory.

It is generally accepted that 5HT acts in the cortex as an inhibitory neurotransmitter (482). This, and the hypothesis regarding the specificity of the circuitry of hypersynchrony, forms the basis of the following attempt to co-ordinate the above findings.

When the cortical serotonergic circuitry is activated, as in non-REM sleep, a specific population of cortical neurones upon which

the serotonergic nerve terminals project, is inhibited; the removal of this population of cells permits, or is associated with the development of the synchrony of sleep in the remaining active neuronal matrix. This state, which displays such synchrony, has also an increased vulnerability to the hypersynchrony of epilepsy. (Theoretically it is just as possible to precipitate an oscillating state by the removal of active elements from a matrix as by adding active components.)

If the non-sleeping animals can be treated in such a way as to mimic the action of the serotonergic projections, and consequently removing the same specific nerve population from the pre-synchronous nerve matrix of the cortex, then one would expect a similar reduction of the seizure threshold. Tryptophan loading may achieve this. Because tryptophan hydroxylase is only located in serotonergic neurones, the increase in central 5HT after tryptophan loading is restricted to physiological sites, i.e. serotonergic nerve terminals, suggesting that the same cells in the cortex will be affected by this treatment as are affected during sleep. Moir (464) established that after tryptophan loading, the increased levels of brain 5HT were proportionately closely related to the normal regional distribution of the amine.

5HTP loading induces a raising of the seizure threshold, although in this case the underlying factors are not identical; as aromatic amino acid decarboxylase is widely located in the CNS, the rise in cortical 5HT after 5HTP loading does not parallel normal 5HT distribution, being considerably less differentiated (464). Grahame-Smith (82) found that tryptophan administration in the rat

could only initiate a state of hyperpyrexia and stereotyped hyperactivity when given in conjunction with an MAO inhibitor, and suggested that MAO might play an important part in determining the functional response of serotonergic neurones to changes in the rates of 5-HT synthesis brought about by altering substrate concentrations. He suggested that 5-HT synthesised in excess of demand and storage capacity would normally be catabolised intraneuronally; only after MAO inhibition would an increase in 5-HT synthesis, after all storage sites had been filled, "spill over" into extra-neuronal release and functional activity. The importance of this idea is that it provides a hypothetical structure of uncoupling serotonergic neuronal activity from substrate concentrations and rates of 5-HT synthesis. However, the fact that tryptophan administration alone can affect the seizure threshold (50, 51, 461, 462) would seem to indicate that this uncoupling may, in certain situations, not be completely effective.

Using tryptophan administration in conjection with an MAO inhibitor, one might then arrive at a situation where an increase in the amount of 5-HT released from serotonergic nerve terminals could be sufficient not only to inhibit the critical population of cortical neurones, but also neighbouring neurones. Alternatively, receptor desensitisation of the critical cortical neurones might develop, which one would also expect to result in a raising of the seizure threshold.

A good illustration of the different effects of serotonergic manipulation is described in a series of papers on the photosensitive baboon, by Meldrum et al. (509, 510), although no explanation is offered. 200-600 mg/kg L-tryptophan, given i.p., slowed background rhythms and simultaneously increased spontaneous paroxysmal activity in the EEG, resembling in these two instances the effects of sleep.

Table A27

Effects of L-tryptophan administration on cortical 5-HT and 5-HIAA ($\mu g/g$ brain) in unoperated, $3\frac{1}{2}$ month old PVG rats

			5HT	5HIAA
A	A	Controls	.45	.42
			.39	.35
			.42	.38

B Experimental; animals given 600 mg/kg L-tryptophan i.p., and subsequently assayed 1 and 2h after the tryptophan administration.

	5HT	5HIAA
1h	.65	.61
	.79	.70
	.77	.74
	.73±0.06(3)	.68±0.05(3)
2h	.79	1.1
	.68	.88
	.83	.95
	.76±0.06(3)	.97±0.09(3)

Large doses of an MAO inhibitor were very effective at suppressing spontaneous paroxysmal activity in the EEG, and also the myoclonic and EEG responses to photic stimulation. Sub-effective doses of the MAO inhibitor, in conjunction with tryptophan loads of 100-150 mg/kg, were equally effective in suppressing epileptic behaviour, as were 5HTP loads.

It is interesting to note in this context that clorgyline, an MAO type A preferential inhibitor, has also been used on the cobalt-implanted rat model and has been shown to suppress epileptic signs in the EEG (J. McQueen, personal communication). I anticipated from the observations reported in the literature and referred to above that by manipulating the serotonergic system in the cobalt-implanted rat a biphasic response would be obtained, namely an increase in spiking associated with increased but still physiological serotonergic activity, followed by a reduction in spike formation associated with the non-physiological synthesis and release of 5HT, and subsequent non-specific cortical inhibition.

EXPERIMENTAL RESULTS

In a preliminary experiment, the effects of a loading tryptophan dose (600 mg/kg) on levels of 5HT and 5-HIAA in the cortex of control, unoperated rats were measured at 1 and 2 hours after loading (Table A27). It had already been established that cobalt-implanted rats responded to a tryptophan load similarly to glass-implanted controls (Table A24, A25). Subsequently an identical dose of tryptophan was given to 30 day cobalt-implanted rats. The ECoG of these rats were recorded before and after the tryptophan administration, and a group of these animals was then given clorgyline (2-4 mg/kg) before a final ECoG recording was made.

Table A28

The effects of clorgyline on cortical 5-HT, 5-HIAA and NAd.

		5-HT	5-HIAA	NAd
A. Controls	1	.39	.33	206
	2	.42	.34	188
		.41	.34	197
B. Clorgyline	3	.54	.20	217
	4	.59	.14	195
	5	.54	.23	199
		.56±.02	.19±.04	204±10

Notes:

1. Figures given µg 5-HT or 5-HIAA/g brain, and ng NAd/g brain.
2. Final values given as $\bar{x} \pm SD'$.
3. Clorgyline given 1h. before killing, 2 mg/kg i.p.
4. Each group of 3 data represent a sample of frontal cortex from 1 rat.

Table A29

Effects of clorgyline on ECoG of cobalt-implanted rats

	Control	After clorgyline
A	23/131	13/82
B	84/157	61/87
C	9/76	4/13
D	121/238	38/116
E	17/84	6/9
F	76/149	27/41

$\bar{x} \pm SD'$: Control: 55 ± 41/139 ± 54

Clorgyline-treated: 25 ± 20/58 ± 40

Notes:

1. All figures represent spikes/min., averaged from a 10 min. recording.
2. x/y represents spike counts from primary/secondary foci.
3. Rats used 10 days post-implant. They were recorded once before clorgyline administration, and again 1h. after 2 mg/kg clorgyline, i.p.
4. Using the paired T-test, it was found that the reduction of spiking in the primary focus was significant at the .05-.025 level, and in the secondary focus at the >.001 level.

Table A30

Spike counts in 10 min. recordings of ECog in cobalt-implanted rats, before and after L-tryptophan and clorgyline

			Time Zero	1h	2h
A.	Controls	1	9/88	21/128	13/70
		2	137/212	79/128	172/282
		3	251/204	197/330	285/357

All controls were given 0.5 ml 0.9% NaCl i.p. after each recording to allow for stress induced in the experimental animals by drug administration and handling.

B. Effects of L-tryptophan, 600 mg/kg p.i., given directly after recording at time zero.

	Time Zero	1h	2h	3h	4h
4	168/177	367/285			
5	97/198	201/267			
6	168/185	237/247			
7	8/98	33/289	27/303	21/248	
8	115/138	212/274	350/311	272/316	
9	34/131	146/223	185/276	181/329	
10	104/87	N.R.	N.R.	403/304	533/508
11	174/195	N.R.	N.R.	325/424	

C. Effects of clorgyline, 2mg/kg i.p. on tryptophan-loaded rats. Tryptophan, 600 mg/kg i.p. given after 1st recording. Clorgyline given immediately after recording at 1h and 3h./...

Table A30 (contd.)

C. Effects of clorgyline, 2mg/kg i.p. on tryptophan-loaded rats. Tryptophan, 600 mg/kg i.p. given after 1st recording. Clorgyline given immediately after recording at 1h and 3h.

	Time Zero	1h	2h	3h	4h
4	168/177	367/285	95/84		
5	97/198	201/267	54/36		
6	168/185	237/247	5/8		
7	8/98	33/289	27/303	21/248	4/8
11	174/195	N.R.	N.R.	325/424	107/93

Notes:

1. Figures given $\frac{x}{y}$ represent spike counts from primary focus (x) and secondary focus (y).
2. In table C, all figures to right of double line represent rats recorded 1h after clorgyline administration.

The ECoG of a control group of animals was also recorded, these being 30 day cobalt-implanted littermates of the first group, given subcutaneous injections of 0.9% NaCl instead of the drug injections. This was to allow for any ECoG effects in the experimental animals due to the stresses of injection and handling. The control group did not display any significant variation in spike counts over the period of observation (Table A30, pt.A).

All rats given a 600 mg/kg dose of tryptophan responded at 1 hour after loading by a 50-100% increase in the spiking rate in both primary and secondary foci. This increase was maintained at 2 h. after tryptophan, and the 4 rats which were recorded at longer times (4 and 5 h.) also showed a consistent elevation of spike counts above normal in both foci (Table A30, pt.B). Then a group of these animals was given clorgyline at a dose that had been previously found to increase levels of 5-HT in the cortex of unoperated control rats by about 30% while leaving NAd concentrations unaffected. (Table A28). This same dose had also previously been found to reduce spike counts in both primary and secondary foci in rats at 10 days post-implant (Table A29).

The clorgyline in this experiment caused a sharp fall in the spiking rates in both foci to levels between 50-90% of the first, pretryptophan recordings. Using the paired t-test, the post-clorgyline spiking rates were significantly lower than the pre-tryptophan rates at the 0.025 probability level (in the primary focus) and at the 0.001 probability level (in the secondary focus). To examine further the effects of manipulation of the central serotonergic systems on the seizure threshold, a group of cobalt-implanted rats was treated with a persistent central 5-HT depleting agent, 5,7-dihydroxytryptamine. At 1 day post-implantation, the experimental

Table A31

Effects of desmethylimipramine and 5,6-dihydroxytryptamine on levels of cortical 5-HT, 5HIAA and NAd

			5-HT	5-HIAA	NAd
A.	Controls	1	.42	.38	177
		2	.38	.32	210
			.42	.35	194
B.	Experimental	3	.24	.19	225
		4	.23	.14	191
		5	.31	.15	203
			.27±.04	.22±.02	206±14

Notes:

1. Figures given as µg 5-HT or 5-HIAA/g brain, and ng NAd/g.
2. Final values given as $\bar{x} \pm SD'$.
3. 10 days before killing, rats were given 5 mg/kg desmethylimipramine i.p., and 1 h. later 50 µg of the free base 5,7-dht. in 20 µl mammalian Ringer's solution i.v.
4. Each group of 3 data represent a sample of frontal cortex from one rat.

Table #32

Effects of desmethylimipramine and 5,7-dihydroxytryptamine on ECog of cobalt-implanted rats.

Days post-implant

	Day 4	Day 10	Day 18	Day 25
RAT A	1/10	6/30	6/12	0/23
B	4/34	5/9	8/13	7/31
C	1/15	2/21	2/10	5/14
D	12/97	3/32	5/9	2/26
E	3/68	0/4	8/53	3/28
F	10/49	7/40	12/13	8/20
G	16/62	15/63	5/27	9/12
H	29/35	67/137	2/2	1/0
I	0/18	5/55	6/31	0/14

$\bar{x} \pm SD'$: Day 4: $8.4 \pm 9.0/43.1 \pm 27.0$

10: $12.2 \pm 19.7/43.4 \pm 37.7$

18: $6.0 \pm 2.9/18.8 \pm 14.7$

25: $3.8 \pm 3.2/18.6 \pm 9.1$

Notes:

1. All figures represent spikes/min., averaged from a 10 min. recording.
2. x/y represents spike counts from primary/secondary foci.
3. 5,7-dihydroxytryptamine and desmethylimipramine given 1 day post-implant.

animals were pre-treated with desmethylimipramine (5 mg/kg, i.p.) a selective blocker of catecholamine uptake, and then at 1 h. given an intraventricular injection of 50 µg 5,7-dihydroxytryptamine, the free base, in 20 µl mammalian Ringer's. Treatment with this combination of drugs has been reported to have a very specific and persistent 5-HT depleting effect in the brain, with only transient changes in catecholaminergic parameters (505, 508). The group of experimental animals was then divided into two, one subsequently being used for biochemical analyses and the other for ECoG recordings.

Cortical levels of 5-HT and 5-HIAA were approximately 40% of normal at 10 days after treatment, while NAd levels were unaffected (Table A31). The effects of central 5-HT depletion on the ECoG proved, however, difficult to evaluate (Table A32). At first sight it would appear that spiking rates are low in the primary focus at days 10, 18 and 25 post-implantation, but the figures quoted here are not outside the wide range of variation of the cobalt-implanted rat model when the ECoG is recorded only for a short (10 minute) period on different days. The absence of any parallel effect on the secondary focus suggested that the reduction of cortical 5-HT levels by some 40% did not markedly alter the epileptic condition. This finding was somewhat unexpected in the light of the theory, already presented, linking raphe tone to the seizure threshold. Possibly the reduction was not large enough to affect the cortical epileptic foci; this seems however hard to reconcile with the observed degree of involvement of the serotonergic system in this model, as instanced by the 30-40% reduction of cortical 5-HIAA (Tables A17, A18, A24).

Table A33

Effects of tolazoline on ECoG of cobalt-implanted rat

Days post-implant:

Day 5	Day 10-11	Day 19-20
$^{1}/_{6} \rightarrow {}^{0}/_{6}$	$^{21}/_{16} \rightarrow {}^{0}/_{3}$	$^{18}/_{39} \rightarrow {}^{14}/_{28}$
$^{3}/_{0} \rightarrow {}^{0}/_{11}$	$^{0}/_{2} \rightarrow {}^{0}/_{5}$	$^{39}/_{47} \rightarrow {}^{29}/_{32}$
$^{0}/_{8} \rightarrow {}^{8}/_{14}$	$^{40}/_{22} \rightarrow {}^{7}/_{5}$	$^{0}/_{1} \rightarrow {}^{0}/_{0}$
$^{3}/_{6} \rightarrow {}^{4}/_{7}$	$^{5}/_{4} \rightarrow {}^{32}/_{15}$	$^{3}/_{1} \rightarrow {}^{4}/_{27}$

Notes:

1. All figures refer to spikes/min., averaged from a 10 min. recording.
2. x/y refers to spike counts in primary/secondary foci.
3. x/y $\rightarrow$ a/b denotes changes in spike counts before and after tolazoline; tolazoline was given i.p., 5mg/kg in saline, immediately after the first recording and 30 min. prior to the second recording.
4. Using the paired t-test, no significant changes were found after tolazoline administration.

In a subsequent experiment, a group of cobalt-implanted rats were recorded before and after the administration of tolazoline (5mg/kg i.p.) at days 5, 10-11, and 19-20 post-implantation. Tolazoline has been reported to be a very potent central 5-HT receptor blocker (Dr. D. Franz, personal communication). This drug did not display any definite effect on spiking rates in either primary or secondary epileptic foci (Table A33), at any stage recorded after implantation. In retrospect tolazoline may not have been the drug of choice for this experiment, as it is also an α-adrenergic receptor blocking agent, and an H_2-type histamine receptor agonist (606). A more specific central 5-HT receptor blocker, N-dimethyltryptamine, has been found to aggravate spiking in the cobalt-implanted rat (Dr. J. McQueen, personal communication).

At this stage the criticism was raised that the changes in spiking rates observed during the tryptophan loading experiment may have been a reflection of diurnal fluctuations in the seizure threshold, this experiment differing from previous ones in that the animals were recorded at different times throughout the day. Schreiber and Schlessinger (52) had already demonstrated diurnal fluctuations of the seizure threshold in the audiosensitive mouse, developing in parallel with the circadian rhythms governing the central serotonergic system. To ascertain the degree of diurnal fluctuation of the seizure threshold in the cobalt-implanted rat, a group of operated rats, 7 weeks post-implant, was recorded at 3 different times during the day. (Table A26). The figures show an apparent decrease in spiking rates in both foci at the two later recording times, but these changes are not significant. (Using the paired t-test, the probability of this reduction being significant are $0.2 > P > 0.1$, for both foci).

Table A26

Mean spike counts/min. in 10 min. recordings of ECoG in cobalt-implanted rats when recorded at different times on same day (50 days post implant)

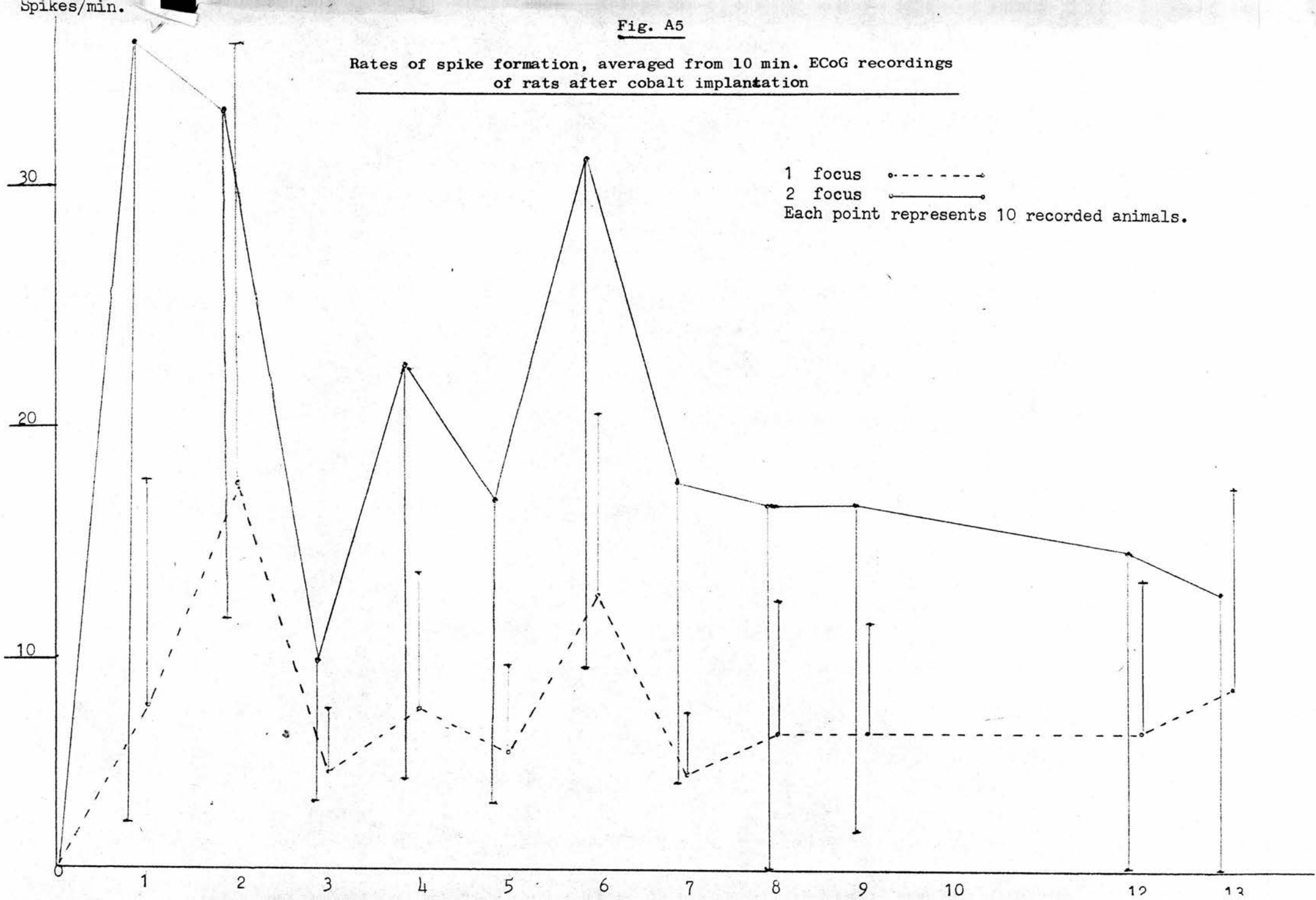
Rat	9.00-10.30 a.m.	12.00-13.30 p.m.	15.30-17.00 p.m.
A	N.R.	0/36	2/5
B	N.R.	7/43	18/37
C	5/23	15/41	N.R.
D	2/7	5/5	N.R.
E	11/13	N.R.	10/11
F	2/21	N.R.	2/39
G	4/8	1/2	2/3
H	25/83	0/3	0/1
I	9/10	6/5	2/10
J	20/27	1/15	2/4
	9.8±8.0/24.0±23.4	4.4±4.8/18.8±17.0	4.8±5.7/13.8±14.4

Notes:

1. x/y represents spikes in primary/secondary epileptic foci.
2. N.R. indicates no recording made.
3. Final values given as $\bar{x} \pm$ SD' (N).
4. The paired t-test was used to compare the recordings; no significant changes were noted (P 0.25).

Fig. A5

Rates of spike formation, averaged from 10 min. ECoG recordings of rats after cobalt implantation



These results are a little surprising in view of the diurnal rhythms found in the audiosensitive mouse (52), but it may simply be the case that the variation in seizure threshold, if it occurs at all in this model, develops before 9.00 a.m. and after 6.00 p.m. They do suggest, however, that the changes in spiking rates found in the drug-treated animals being recorded for periods of up to 3 to 4 hours (Table A30, pt. A and B), could not be ascribed to diurnal changes in the seizure threshold as the experiments were all carried out between 9.00 a.m. and 6.00 p.m.

In a final attempt to link developments in the ECoG of the cobalt-implanted rat with the biochemical findings, a group of 10 male PVG rats, 2.5 months old and weighing 225-275g, was given cobalt-implants and subsequently recorded once weekly for just over 3 months. In an attempt to reduce the degree of experimental variation, care was taken to standardise recording conditions as follows; all animals were recorded in a set sequence at a standard time of day, and to allow for the stress induced by handling the animals and placing them in the novel environment of the recording cage, a period of 10 minutes was allowed between electrode connection and recording.

The ECoG recordings were processed simultaneously onto paper print-out for visual inspection, and onto recording tape for subsequent computer analysis by the program of Hill and Townsend (567). Two distinct peaks of spiking appeared over the 3 months, the first developing during the first week post-implant and falling off to 20-25% of peak values between 14 and 21 days post-implant, and the second peak developing at weeks 4-7 (Figure A5). After the second peak the rates of spiking appeared to stabilise.

The first increase in spiking develops at roughly the same time as the reduction in the activity of cortical catecholamine-related enzymes discussed in this thesis, and the fall in the activity of the synthetic enzymes of acetyl choline and GABA (466). The next phase, a major reduction of spiking rates between 14-21 days, develops while concentrations of 5-HIAA in the cortex and the activity of tryptophan hydroxylase in the raphe are also becoming reduced.

Between weeks 3-5, some of the rats display a relatively quiet ECoG, and there is evidence that during this period some recovery of cortical cholinergic and gabaminergic fibres is taking place (466). Beyond this time correlations between events in the ECoG and biochemical changes appear more tenuous. One can only formulate hypotheses as a basis for further experimentation. For example, the second increase in spiking at 6 weeks may be due to denervation hypersensitivity phenomena as the injured fibres compensate and/or regenerate in the affected areas, while the period between weeks 7-13 could represent a 're-wired' steady state subsequent to the recovery of the catecholaminergic and serotonergic parameters.

SUMMARY OF THE PHARMACOLOGY SECTION

We are left with a series of data that do not match complately; but after allowing for variabilities introduced by factors such as species differences and the non-specificity of certain of the drugs used to manipulate the serotonergic systems, certain broad tendencies emerge. The relationship between serotonergic activity and the seizure threshold appears to be triphasic. In general, a reduction of serotonergic tone is associated with a lowering of the seizure threshold. A slight increase in serotonergic tone may have the same effect, and a

large increase is associated with an elevated seizure threshold.

It should however be stated that the data generated by the tryptophan loading and clorgyline experiment is open to other interpretations than the one offerred above, that of excess cortical 5-HT causing a general cortical inhibition. It is also possible that the excess 5-HT causes a desensitisation of the cortical cells that the raphe projects to, thereby preventing the "damping" elements from being removed. Another alternative is that a build-up of tryptamine and/or other amines normally catabolised by MAO type A may contribute to the reduction in spiking after clorgyline.

If it is true that a moderate stimulation of the serotonergic system can exacerbate epileptic behaviour, then it becomes apparent that the clinician using drugs designed to elevate levels of 5HT in the brain (as in the treatment of myoclonus), may be faced with the problems of dealing with two thresholds in his titration of the patient. A rise too small may be counterproductive, and an excessive increase causes non-specific sedation. As dietary tryptophan is difficult to monitor outside the clinical or laboratory context, it may prove difficult to maintain optimal tryptophan levels in non-hospitalised patients. However, the success of various clinical trials, already discussed, suggests that the room for manoeuvre between the two thresholds is great enough to make this form of anti-convulsant therapy useful. And it is not as if the sedative side-effect at high doses is too high a price to pay, as it is in any case a side-effect of standard anticonvulsant overdose; indeed the long-term problems of serotonergic manipulation are probably less ominous than those of, for example, diphenylhydantoin, where there are

reports of Purkinje cell fall-out after chronic use of the drug (586), although these findings are disputed (585). Nor does the risk of drug habituation seem likely, as can occur with the chronic use of phenobarbitone.

In addition to the biochemical and pharmacological approaches, another method of analysing events in the cobalt model would be to study those aspects of behaviour in the rat which are thought to be influenced by serotonergic tone. For example, it might prove rewarding to examine sleep patterns in the cobalt-implanted rat, for a loss in serotonergic tone may be reflected here as a reduction in sleeping time. Alteration in body temperature control might also be worth studying.

CONCEPTS OF NEURONAL SYNCHRONY

A hypothesis is developed concerning the role of the raphe in epilepsy.

A summary of the available data linking 5HT metabolism to the seizure threshold has already been presented. To develop the hypothesis concerning the role of the raphe system in epilepsy, it is necessary to disgress a little and include a short section on the effects of 5HT on cortical arousal, indicating what this means in terms of the behaviour of individual neurones in the cortices, and of the overall functioning of the cortices themselves.

Let us begin by describing all states of the CNS in terms of the efficiency of data handling; several concepts at once follow. In the alert individual EEG tracings display complex and shifting wave forms. If the eyes are closed and the mind relaxed, characteristic rhythms develop over different areas of the cerebral cortices, the

well-known alpha-rhythm appertaining to the occipital areas, for example, and the 11-12 Hz μ-rhythm of the motor cortex. These rhythms appear to denote the resting state of the cortical areas involved. If cortical input is increased by opening the eyes, or if intracortical activity is increased such as by the request to carry out mental arithmetical operations, these resting rhythms are disrupted and the EEG again returns to the more varied waking form.

We can examine the other end of this scale by monitoring the EEG of a patient being brought under anaesthesia. Excepting the initial excitatory phase, the deepening stages of anaesthesia can in fact be characterised by the gradual reduction of information, or variation, in the recording. This presumably reflects both the decrease in cortical input, and also in intra-cortical activity. Consciousness returns as the data-handling capacity of the brain, primarily the cerebral cortices, increases to normal values. In the context of the developing argument I would like to define consciousness here as a parameter of date-handling.

One would expect that there must be a constant evolutionary pressure on animals to develop rapid and efficient machinery for handling the data in the input from a varied and varying environment. One of the limiting factors of the capacity of a neuronal machine is the velocity of neuronal conduction, and it is of interest to note here that this factor is found to correlate well with the degree of evolutionary advance in many species. The speed of maximal information flow in nerve cells in the jellyfish is of the order of 10cm/sec, the best figures for worm nerve cells is around 100cm/sec, while insects and anthropoda display rates of conduction of up to 1 metre/second. The anthropoidal neurone can conduct at rates of up to 10m/sec, and

in man the fastest neuronal rates are around 30 m/sec. (326).

Other limiting factors would include the degree of neuronal arborisation and the actual number of neurones. These determine the width of information channels and the possible ranges for information coding and permutation. A further factor would be the temporal density of signals that can be transmitted throughout a circuit before interference and loss of signal develops. This is in turn derived from the conduction velocity, the refractory period, and under certain circumstances the time-scale of synaptic chemistry.

The state of alertness or arousal may be regarded as the resultant state, or system, of a number of sub-systems in dynamic equilibrium. These sub-systems could be specified biochemically, anatomically or functionally: these three types of classification may overlap, but are not necessarily synonymous.

The working brain can be manipulated in terms of any of the above types of sub-system; biochemical sub-systems may be manipulated pharmacologically, anatomical sub-systems by the use of local lesioning or stimulating electrodes, and functional sub-systems by the technique of manipulating physiological input.

At this stage I wish to introduce a further concept, "electrical fluidity". Electrical fluidity describes the extent, as measured in terms of the number of neurones involved, to which a neuronal event occurring in a neuronal matrix will spread within that matrix, in a given time.

We can also define the range of this factor in terms of electrical behaviour of a nerve net. At very low, or zero, values of electrical fluidity each neurone becomes isolated; no information

passes from cell to cell, and the data handling capacity of the system is zero. At pathologically high values of electrical fluidity, an electrical event occurring at any point in a nerve matrix is transmitted throughout that matrix, and every cell will be stimulated by every other cell. It is then impossible to separate groups of neurones in terms of specific function, all compartmentation of information has broken down, and the data handling capacity of the system is again zero. At intermediate values of electrical fluidity, we have a state wherein one neurone can communicate with another, and the co-ordinated functioning of groups of nerves is possible, while at the same time the degree of compartmentation is such as to afford the processing of unrelated data concurrently.

This intermediate state is that of the normal working brain during arousal; the hypothetical state of hypofluidity is in fact found in the case of barbiturate intoxication, and a hyperfluid state is displayed for example in epilepsy with its typical breakdown of compartmentation of function, or in the state of spreading depression, and possibly in the states of synaesthesia and hallucination. The electrical fluidity of a nerve network can also be manipulated by such techniques as kindling, where the seizure threshold is gradually reduced by a course of sub-convulsive thalamic shocks (327). It may also be the case that the learning process and memory consolidation are the result of a controlled increase of electrical fluidity in specific circuits, but this is outwith the scope of this thesis.

There is, therefore, a degree of electrical fluidity optimal to cortical function, above or below which data handling is impaired.

It is also appropriate here to discuss some of the prevailing theories of neuronal interaction, specifically the category of neuronal synchrony.

Various mathematical models have been constructed in attempts to explain aspects of the behaviour of the CNS. One project, perhaps the earliest, was developed by Selfridge (311). His model consisted of a network of units all possessing the following characteristics; first, a uniform speed of conduction, second a refractory period, and third an "all-or-none" type of firing response. His model was in fact designed to investigate the transmission of electrical information in the myocardium, but could equally be applied to the study of the brain. He found that in such a conducting network, certain types of stimulus could initiate rhythms of electrical activity synchronising the firing of large groups of units. These rhythms could be damped by the introduction of new inputs, provided these were not in phase with the standing rhythms, and strengthened by a reduction of input, or by further in-phase input. The procedures tending to strengthen standing rhythms could be exemplified by the effects of eye-closure on α-rhythms already mentioned, and the form of epilepsy known as flicker-epilepsy induced by specific rhythms of visual input. These could be taken as representing a decrease in input, and the superposition of an in-phase input respectively.

The stimulation of various sub-cortical areas, including the nucleus tractus solitarius (513) or raphe nuclei, and certain thalamic nuclei, can also induce cortical synchrony. The cortical synchronies developed in the four stages of non-REM sleep, and which are used to characterise each stage, are partly generated by a direct synchronising tone of the raphe itself; however, the reduction in cortical

input is also critical. Koella and Czicman have demonstrated that raphe activation causes an inhibition of activity of the reticular activating system (RAS) (307), so the raphe exerts a dual effect on the cortex's tendency to synchrony. In addition, the abolition of visual input is probably also important. The RAS itself generally exerts a desynchronising tone on the cortex; stimulation of the RAS in cats has been reported to cause increased vigilance, with an increase in sensitivity to stimuli of all sorts (424). Conversely lesions of this system result in a state of hypersomnia and cortical hypersynchrony (Lindsley, 422). The actions of the raphe and RAS on the cortex then appear to be diametrically opposed, and the state of cortical arousal at any one time will be a reflection of, amongst other things, the balance of raphe and RAS tone.

It is also interesting to note here that the RAS, in desynchronising the cortex, increases the data-handling capacity. A novel stimulus, which arrives at the cortex, is firstly recognised as novel, and then a train of events develops known as the orientating reflex; the cortex sends information to the RAS which responds by amplifying this signal and relaying it back to all areas of the cortex resulting in the general arousal which can be caused by apparently non-intense stimuli (303).

Flicker epilepsy has been mentioned above as an example of reinforcing input creating synchrony in a neuronal matrix. Strobe illumination is not only effective at awakening existing epileptic foci, but also elicits epileptiform EEG abnormalities in 4 per cent of non-epileptic subjects (546). This has been interpreted by some workers to mean that the safety margin between normal and epileptic or

oscillating modes of brain function may be relatively slight (549).

In the design of complex serve-mechanisms, unintended and destructive oscillation is recognised as a significant hazard. The brain is by its complexity vulnerable to this type of malfunction, as oscillatory behaviour could in theory develop at any level from the simple circuit to three-dimensional nerve matrices. If this view is valid, it indicates that attempts to isolate one malfunctioning unit as the cause of the oscillation are doomed to failure, as the oscillation is rather the property of the oscillating system. Analogously, as epilepsy could refer to oscillations in any of an infinitely wide range of levels of circuit, this would seem to indicate the inadvisability of attempting to manipulate sub-systems selectively. It is interesting to note here that the most potent anti-convulsants, as mentioned earlier, have a very wide range of effects; indeed their efficacy may depend on their spectrum of actions on a wide range of sub-systems. Alternatively, one could postulate that all the anticonvulsants exert, amongst their other effects, one action on some property common to all sub-systems, for example the transmission of the action potential.

In situations where the overall system is the product of a dynamic equilibrium of sub-systems, any relationship between the functioning of the system and that of one of the sub-systems need not be a first-order one. Each sub-system may operate over a wide range, only certain values of which are optimal in terms of the functioning of the system, performances above or below this range resulting in a deterioration of performance of the system. This may explain findings by Kellog (512), that both increasing or reducing central

amine levels had the effect of raising seizure threshold.

The more complex the arrangement of sub-systems, the greater the redundancy in those sub-systems, the greater become the possibilities of absorbing and adjusting to changes in the input to, and via, the various sub-systems. However, this is offset by another rule of systems behaviour derived by Ashby (323), who stated that a system tends to be dominated by its least stable sub-system. And this suggests that in certain epilepsies a specific therapy may in fact be useful - for example, in the model of the audiosensitive mouse, where the low seizure threshold has been associated with a deficient serotonergic system, treatments to raise central 5HT levels have proved very effective at raising the seizure threshold. Interestingly enough the diurnal fluctuations of the threshold in this model have been shown by Schneider and Schlessinger (52) to follow the circadian rhythms of the 5HT system; of all the known central aminergic neuronal systems this appears to be the least stable, NAd and DA systems showing no such involvement.

I would like to suggest that the neuronal behaviour involved in generating the epileptic condition can be described as a consequence of a pathological increase in the electrical fluidity of the areas of brain involved. Again let me cite the case of kindling, where the epileptic condition appears to be a result of the gradual increase of electrical fluidity during the kindling treatment.

If rats are given daily shocks in the amygdala at current levels high enough to produce an after-discharge but too low to elicit any motor response, this procedure over a period of 1-2 months encourages the progressive development and exacerbation of stimulus-induced

epilepsy. As the procedure continues the electrical resistance to spread decreases until, at 6-7 months, a condition is produced characterised by spontaneous motor seizures (327).

At this stage in the discussion it becomes necessary to return again to the relationship between central 5HT systems and the state of cortical arousal, or the data handling capacity of the cortices, a parameter which I have already suggested is linked to the factor, electrical fluidity.

Jouvet and Pujol (511) have shown that intracaretid injections of 5HT in the cat cause a marked cortical hypersynchrony, and sedation. The increased synchrony, as we have already explained, indicates a reduction of the temporal and spatial variation in the neuronal matrices of the cortices, and therefore a reduction in data-handling capacity - hence the sedative effect.

The hypersynchrony is probably mediated by cells in the area postrema, as the hypersynchrony and sedation can also be elicited by topical application of 5HT to this area (337, 338, 511). Fibres can be demonstrated leaving this area, entering the nucleus tractus solitarius; appropriate stimulation of this tract will also cause cortical hypersynchrony and sedation (339, 513). A severe state of insomnia can be precipitated in the rat by the tryptophan hydroxylase inhibitor, para-chlorophenylalanine: this insomnia is reversed by the oral or intravenous administration of 5-HTP, which is metabolised centrally to 5-HT in the depleted but otherwise undamaged serotonergic neurones (594).

Some delicate electrophysiological studies have shown that individual cells in the raphe are active during non-REM sleep, but

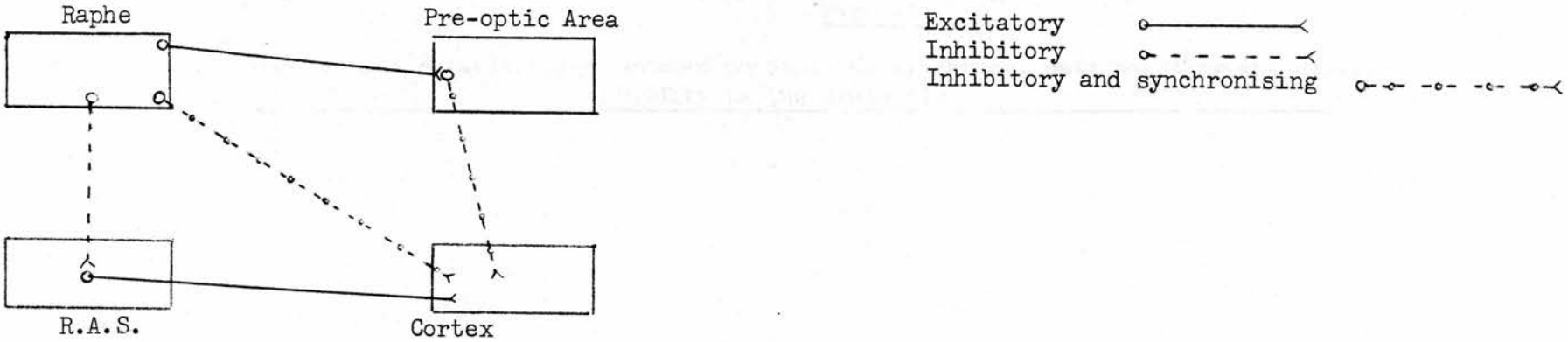


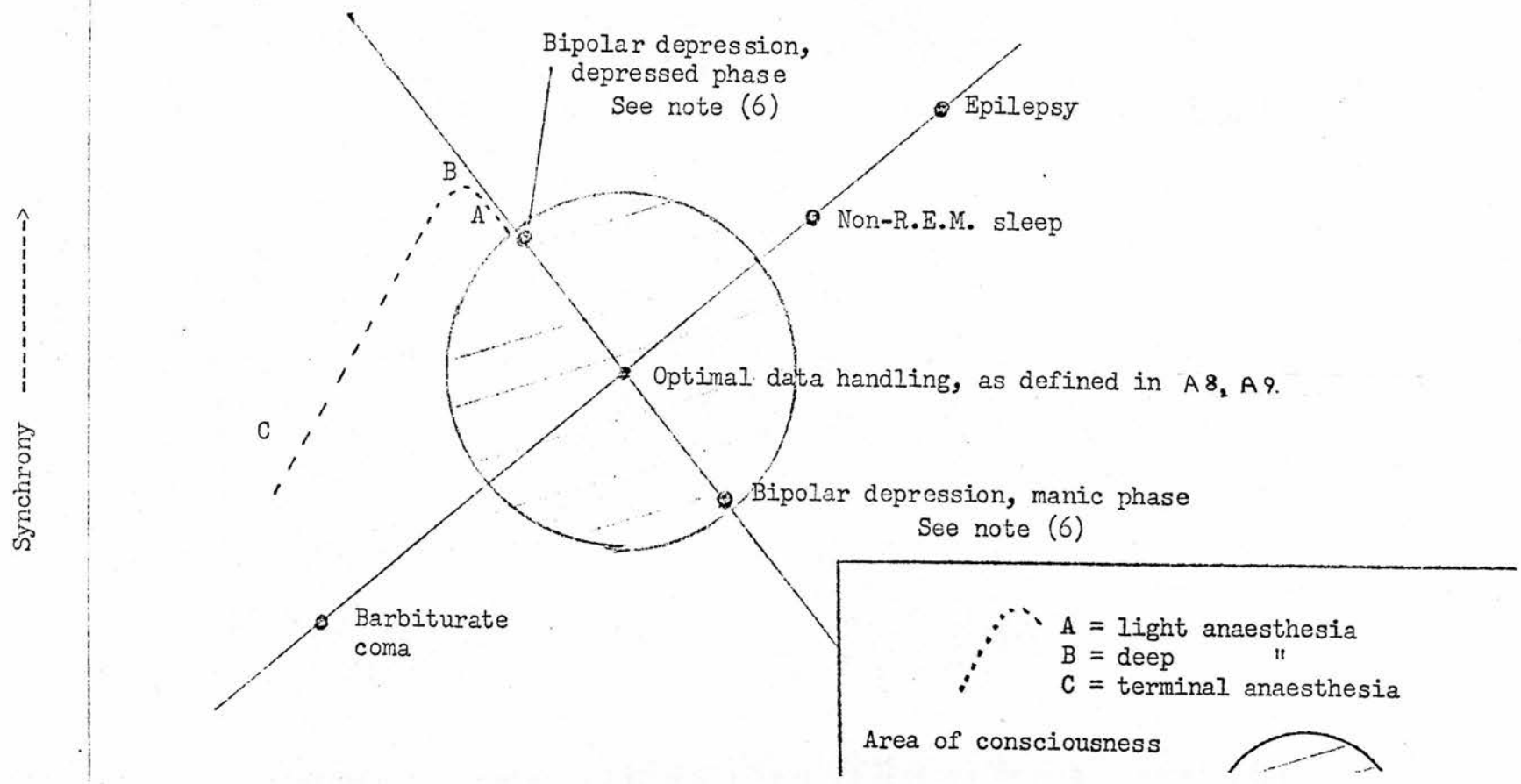
Fig. A7

Theoretical relationship of extracortical structures to the seizure threshold

Fig. A10

Theoretical relationships between concepts of synchrony, data handling and electrical fluidity in the brain (3)

(3)



inactive during waking and REM sleep (340). Further evidence of the role of 5HT systems in determining the state of cortical arousal is provided by cortical perfusion studies; sleep, or states of cortical depression are associated with increases in cortical 5HIAA production, and increased cortical 5HT levels (52).

These data all suggest that the activity of the serotonergic system has a direct influence on the degree of cortical synchrony, and the data-handling capacity of the cortices. It must be admitted that it is not always possible to guarantee the specificity of various pharmacological treatments; for example para-chlorophenylalanine (PCPA) as has already been described, also inhibits tyrosine hydroxylase, and may in consequence lower the tone of NAd and DA systems as well as of 5-HT systems. Furthermore, it would probably be a mistake to think of the serotonergic system (namely the raphe nuclei and their projections) as either biochemically or functionally homogenous. For example, certain nuclei within the raphe are more sensitive than others to the 5-HT-depleting action of para-chloroamphetamine (PCA), notably nucleus B9 (282). There is also a report (283) of preferential degeneration of the B9 nucleus after PCA. Similarly, although the projections of the various anterior raphe nuclei reach all areas of the cerebral cortices, the degree of involvement of each nucleus may determine the areas of cortex involved. Although the effect of raphe stimulation on all identified post-synaptic cells has so far been found to be inhibitory (552), the selective withdrawal of specific units from a nerve matrix could exert a wide range of effects.

A part of the data presented above is assembled in figure A7 and A8-10. The pictures are not complete - for example, NAd systems are

also involved, as sectioning the noradrenergic dorsal bundle has been shown to increase cortical and raphe 5HT synthesis, with consequent hypersynchrony and hypersomnia (511). Other systems may be involved. Figure A10 shows how the factors cortical synchrony, data-handling capacity, and electrical fluidity (E.f.) are related.

The following further comments regarding these figures may be made.

G. Hill (personal communication) finds that analyses of EEG recordings of cobalt-implanted rats show that spikes develop most frequently from the rising (surface negative) phase of synchronous events, thus indicating that the pathological synchrony is in some way facilitated by specific aspects of apparently normal synchronous behaviour.

The basic form of Figures A8 and A9 is obtained as follows:- at the theoretical value of zero E.f., when all neurones are effectively isolated, no information can be processed by the nerve net. At maximal values of E.f. when all electrical events are transmitted through the entire net, compartmentation breaks down and again data-handling is reduced. The state of optimal data handling must represent therefore a value of E.f. somewhere between these two conditions, and the factor E.f. has been defined with this in mind.

Using the above definition of E.f., it becomes possible to justify the inclusion of manic and hallucinatory states in the graphs. Hallucinations if one accepts a circuital basis for information storage, represent an abnormal processing of data, an activation of inappropriate and normally unrelated circuits, and therefore an alteration in the spatial co-ordinate of the E.f. value.

Mania and depression on the other hand are also represented in these figures as states associated with altered E.f. values, but in these states the temporal co-ordinate of E.f. is probably more important.

Of the two states depression is perhaps the better documented. Firstly there is some electrophysiological evidence that at least certain types of information processing are slowed; Shagass and Schwartz (555) have found that the refractory period between stimuli in the sensory evoked response model is significantly greater in depressed patients, indicating a reduced temporal component, and this is perhaps one of the factors underlying the findings of a decrease in mental and psychomotor speed in bipolar depressives (556), and the subjective feelings of slowness, retardation and inhibition associated with depression. Bipolar depressives also appear to display a more highly synchronised EEG when in the depressed phase (557).

The literature concerning mental and psychomotor performance in mania is contradictory. Kraepelin (558) reported an increase in work and mental performance in the early stages of mania, with performance falling as the mania increased; this is similar to improvements found by Weiss and Laties (559) after small doses of amphetamine. However Blackburn (556) found no significant increase in mental and psychomotor performance in manic patients. The available neurophysiological evidence is scanty. Dr. A.I.M. Glen (personal communication) has found that the rate of peripheral nerve conduction is slightly elevated in manic patients, and returns to lower, normal values if lithium is administered, the change occurring in step with the clinical improvement.

Again assuming the circuital basis of information storage, this suggests that the temporal co-ordinate of E.f. may be elevated in mania. The manic phase is associated with a relatively desynchronised EEG (557).

Not only the extent of synchrony, but also the particular rhythms involved, are apparently associated with various states. The two factors are probably connected; G. Hill (personal communication) has suggested that the frequency of a synchronous field is in part determined by the number of active units in that field, such that an inverse correlation exists between frequency and number of neurones involved.

In mania the reduced synchrony is associated with a downwards shift in the dominant α-rhythm frequency (557), and this has also been found in ethanol-induced stupor (560). Conversely, an elevated α-rhythm is found in depression (557) and the hallucinatory state elicited by LSD (561).

It must be noted that, like the electrical fluidity factor, the 'synchrony' axis as used here is only quantitative, and cannot distinguish between the normal synchrony of sleep and the uncontrolled synchrony of epilepsy. These two states are patently different with respect to both the underlying biochemistry, and circuitry. The diagrams are therefore merely descriptive, and not suitable material to base predictions on.

In the condition of epilepsy it is generally assumed that the 'spike' represents the summated response of a large population of damaged or affected neurones firing in pathological synchrony. In the healthy animal, as we have seen, the tendency for cortical cells to develop synchrony is affected by various factors including the activity of the raphe with its wide-spread projections to all parts

of the cortex. The cobalt rat for example, when placed in the recording cage, displays an initial phase of exploratory behaviour. Then if left to itself, it becomes quiet, sleepy, may even fall asleep, and it is during this stage that spikes tend to appear, or become most prominent. If the rat is aroused the incidence of spiking falls. This may be analagous to that group of patients who display an increased tendency to developing fits when drowsy, just before sleep (305, 306), another argument that tendency to cortical pathological synchrony is affected by raphe tone. In fact 'sleep' epileptics are reckoned to constitute 45 per cent of all Grand Mal patients (398, Janz) although other workers find smaller percentages (516). The site of the focus appears to be important in determining whether the epilepsy is 'sleep', 'awake' or 'random'. Frontal foci tend to be associated with 'sleep' epilepsy, parietal foci are associated with 'random' epilepsies (398). In fact the degree of raphe tone appears to be quite critical; sleep epileptics display two peaks of fits during sleep, the first just after falling asleep, the second occurring during early morning sleep (399, 400).

Even more specifically, an analysis of the incidence of fits during different stages of sleep reveals that fits tend to develop only in stages 2 and 3, not in stages 1 and 4; in other words some synchronies are more prone to epileptic triggering than others.

A few experiments have also been done in epilepsy models which broadly agree with these findings. For example, Cohen et al (535) have established that the electroconvulsive threshold in the cat is at its lowest during slow-wave sleep; and in the cobalt focus in the rat, Colasanti et al. (536) demonstrated that, with the exception of

the period of continuous spiking, seizure activity was maximal at the beginning and at the end of non-REM sleep episodes. Wyler et al. (551) have reported an elegant series of individual cell recordings in the chronic alumina focus in undrugged monkeys and have shown that cells that behave as epileptic cells by firing in bursts do so for longer and at a higher frequency during sleep, and that sleep spindles which are associated with doublet or triplet firing in normal cortical neurones, were shown to elicit exaggerated bursts in the epileptic neurones.

Finally, and very significantly in the light of the description of epileptic behaviour as a pathological extension of synchrony, G. Hill (personal communication), after performing various types of analysis of EEG recordings from cobalt-implanted rats, finds that the spike is most likely to occur from the rising component or the peak of a minor synchronous event.

In many patients synchronised sleep acts as a convulsant; as the cortex develops slower rhythms, the appearance of generalised bilateral hypersynchronous behaviour increases (314, 315, 316). Some focal epilepsies are also aggravated (314, 317). Desynchronised REM sleep, on the other hand, is associated with the abolition or reduction of various focal and generalised bilateral epileptic discharge patterns (318). It is also suggestive that workers in this field have established that the tendency of discharges to spread, in other words the spatial component of electrical fluidity, is also affected by the different types of sleep synchrony. Perria et al. 1966 (328) and Schwartz et al., 1964 (317) both established that during synchronized sleep, the interictal discharges from focal epilepsies extended over a

wider area of the cortex than during wakefulness, spreading in some cases to the secondary focus. Conversely, during desynchronised REM sleep, interictal discharges display maximal localisation. In this case therefore electrical fluidity appears to vary directly with the degree of synchrony. Other workers have noted the similarities between the EEG activity of sleeping brain and epileptiform discharges. Both are considered to be manifestations of widespread, synchronous, episodic neuronal activity (Creutzfeldt et al. 1966, 382), possibly sharing brain mechanisms for diffuse distribution. Skinner and Lindsley (383) and Juko et al. (384) showed more recently that it was possible to suppress sleep spindles unilaterally by ipsilateral lesions of the rostral thalamus and underlying inferior thalamic peduncles. If these lesions exert their effect on sleep spindles by reducing the propensity of thalamic and cortical neurones to become involved in synchronous discharge, they may also influence epileptic behaviour.

Feeney and Gullotta, 1972 (381) used a similar preparation of a unilateral rostral thalamus lesion to examine the effects of such treatment on the seizure threshold. They found that after infusing pentylenetetrazole to the point of seizure, the frequency of discharges in the cortex ipsilateral to the lesion was significantly lower than on the contralateral side. This was not apparently due to a general cortical depression as evoked stimuli to light were not altered.

Focal epilepsy in penicillin-induced foci was also inhibited on the cortex ipsilateral to the lesion; and there are two reports in the literature on the clinical use of this operation, both of which report improvement and some cases of complete suppression of clinical

seizures in Petit Mal and Grand Mal. (Mullen et al., 1967, (385), Spiegel et al., 1951 (386)).

These lesions may act by altering the functional state of the remaining thalamo-cortical circuitry; they suppress the excitability of non-specific thalamic nuclei which are considered important for the wide-spread co-ordination of synchrony (Anderson and Manson, 1971, (387), Purpura, 1970, (388)). More recent work by Feeney and Gullotta (534) has centred around the use of high frequency stimulation of the caudate nucleus and they find that this treatment in the cat has a generally desynchronising effect on cortex and suppresses sleep spindles and also interictal discharges from cortical penicillin-induced foci.

The cortical recruiting response, another index of cortical electrical fluidity, produced by the stimulation of various thalamic nuclei, is also abolished by the desynchrony of REM sleep (319 to 321).

The concept of different cortical states being more or less conducive to epileptic behaviour may have clinical implications. Sterman et al. (537, 538), using biofeedback techniques, have trained cats to develop sensori-motor rhythm (SMR) (12-16 Hz), and shown that these animals have a significantly raised seizure threshold to hydrazine with respect to control animals; cats trained to suppress the SMR displayed a reduced seizure threshold. A clinical follow-up has been published; Sterman et al. (539) showed that various epilepsy patients, who were selected on the criterion that they were unamenable to drug chemo therapy, developed a significant reduction in fits after using biofeedback operant conditioning to increase SMR activity.

CONCLUSION

The raphe is, as we have seen, capable of exerting both directly and indirectly, a powerful synchronising tone on the cortex. It can also vary this synchronising tone; it is not just an on/off control but a range of control which is displayed in the four stages of sleep. It seems not unreasonable to suppose that the raphe can also respond to the degree of cortical synchrony, for in all physiological situations of an effector link operating between an effector organ or system and a target organ/system, a feed-back loop operates, involving a monitoring apparatus that feeds information back to the effector so that it has some way of maintaining its output at an efficient or physiologically appropriate level. If the raphe can respond to the varying degrees of cortical synchrony, can it also respond to the pathological synchrony of epilepsy?

The results discussed in the 5HT section indicate that the raphe might be doing just this. The changes in cortical 5-HIAA concentrations and in tryptophan hydroxylase activity in the raphe appear to develop at too late a stage in the cobalt-implanted rat to be associated with the development of the epileptic foci, and in this respect the response of the serotonergic system differs from that of all the other systems so far examined in the cobalt-implanted rat. The fact that the cortical changes develop bilaterally also suggests a response of the serotonergic system as a whole, rather than a local involvement of serotonergic nerve terminals associated with the foci.

To examine the possibility of the raphe acting "protectively" in the cobalt-implanted rat, and possibly in other forms of epilepsy, it would seem logical to continue the work reported in this thesis by examining the effects of electrical stimulation of the raphe, and surgical lesions of the raphe, on the tendency of the cortex to develop epileptic signs.

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NOTE

On pages 262 and 263 reference is made to a 'regrowth' of NAd fibres; this was originally meant to provoke discussion at the meeting; the biochemical changes can be more easily explained by changes of activity in already existing neurones, rather than an ingrowth of new fibres.

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Changes in Monoamine-Related Enzymes in Cobalt-Induced Epilepsy

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The evidence that points to the involvements of aminergic neurons in epilepsy can be divided into two sets. First, the work that indicates that drugs acting on amine metabolism can modify seizures, including data on the use of reserpine to lower brain amine concentrations in animals (Schlessinger *et al.*, 1968) and man (Pallister, 1959), and *p*-chlorophenylalanine to decrease brain 5-hydroxytryptamine in rats (De La Torre & Mullan, 1970). These studies all demonstrated that a general decrease of amines tended to increase susceptibility to epileptic seizures. This has been the subject of a review by Carlsson (1974).

Conversely, drugs that potentiate the activities of brain amines, including monoamine oxidase inhibitors (Prockop *et al.*, 1959) or tricyclic anti-depressants (Lehmann, 1967), have at least moderate protective effects. Recently this series of data has been refined into an analysis of the roles of the different amines; dopamine (3,4-dihydroxyphenethylamine) and noradrenaline are now thought by some workers to exert opposing influences on an epileptic focus, inasmuch as dopamine and its agonists can be shown to decrease 'spiking' in the cobalt model, whereas noradrenaline agonists have the reverse effect (Ashcroft *et al.*, 1974).

The second set of information consists of work that indicates that an alteration in the concentrations of brain amines, presumably parameters of a functional change in specific cortical circuits, occurs in the development of audiogenic seizures (Schlessinger *et al.*, 1967) and in cobalt epilepsy (Emson & Joseph, 1975). However, other workers have found no changes in brain amines related to epilepsy (Hansen *et al.*, 1973), so the case cannot be said to be by any means complete.

In an attempt to clarify the problem of amine involvement in epilepsy, a study was undertaken of the following amine-related enzymes in the cobalt model (Dow *et al.*, 1972): tyrosine hydroxylase, catecholamine *O*-methyltransferase and monoamine oxidase. γ-Aminobutyrate aminotransferase was also investigated. The purpose was to use these enzymes as cell markers to study the integrity of aminergic circuitry in the cortex around a cobalt focus, and to ascertain whether these specific neurons could be demonstrated to react differentially.

Tyrosine hydroxylase was assayed by the method of Hendry & Iversen (1971), it being assumed that this would be the most specific marker for noradrenaline neurons, and the catechol *O*-methyltransferase (Axelrod & Tomchick, 1958) and monoamine oxidase (McCaman *et al.*, 1965) assays were mainly corroborative. γ-Aminobutyrate aminotransferase was examined (Hall & Kravitz, 1967) in the hope that it would provide data on the integrity of local inhibitory gabaminergic fibres and their postulated role in the eventual suppression of a focus, and also as an index of the glial reaction that is associated with the cobalt implant.

Histological studies of the cobalt model done in our laboratory (Emson & Joseph, 1975) by using the Fink–Heimer (Fink & Heimer, 1967) technique have demonstrated degenerating terminals, axons and cells around the primary lesions after 4 days, extending to the 29th day after implant, at which time spikes are still present in the electrocorticogram. Other enzymes so far studied, including glutamate decarboxylase, choline acetyltransferase and aromatic amino acid decarboxylase, tend to follow a similar pattern, reaching their lowest activities in the primary (1°) focus at around day 8 and re-

covering at 24 days. It is probable that these changes, which are reflected in the secondary (2°) focus to a lesser extent, represent a specific neuronal involvement; lactate dehydrogenase, a cytoplasmic enzyme, is only decreased in the lesion area, and is not a part of the pattern of 2° focus development at all. The rate of spiking is inversely correlated with these findings, reaching a peak at days 8–12 and decreasing towards day 24, and probably reflects the enzyme picture (Emson & Joseph, 1975).

Of the enzymes studied, only γ-aminobutyrate aminotransferase returned to normal activity at around day 30 (Fig. 1*a*); the peak activity of γ-aminobutyrate aminotransferase occurring in the 1° focus, and to a lesser extent the 2° focus, at around day 9, probably indicates the intense glial reaction that is developing at this stage.

The pattern of changing γ-aminobutyrate aminotransferase activity agrees well with those studies of enzymes already carried out, and with the general time-course of cellular degeneration (Emson & Joseph, 1975). However, tyrosine hydroxylase activity (Fig. 1*b*) is still low at 30 days, at only 30% of control values. Monoamine oxidase and catecholamine *O*-methyltransferase activities are also low, at 20% and 40% respectively of control value. All these enzymes appear to have returned to normal by days 75 and 100, and the fact that they all share a similar time-course, and at 30 days are proportionately very similarly affected (20%, 30% and 40%), could indicate that noradrenaline neurons do not regrow into the area of an epileptic focus at the same rate as, for example, cholinergic neurons or cell regeneration in general (Emson & Joseph, 1975).

These results could be due to either an increased susceptibility to Co^{2+} or a genuinely slower rate of repair; the fact that this pattern is also found in the 2° focus, where Co^{2+} is probably not involved, tends to support the latter.

The rate of spiking in a 1° or 2° cobalt focus, followed over a period of some months, tends to form a distinct pattern. Firing rates reach a peak at 8–12 days and then decrease over the next 20 days, until at day 30 they are at 10–15% of the peak values. After day 30, spiking continues to decrease, though less rapidly, over a period of several months. It could be that this apparently biphasic response represents an initial intracortical phenomenon during the first month, involving cholinergic and gabaminergic fibres in the suppression or resolution of the focus, followed by a slower regrowth of aminergic

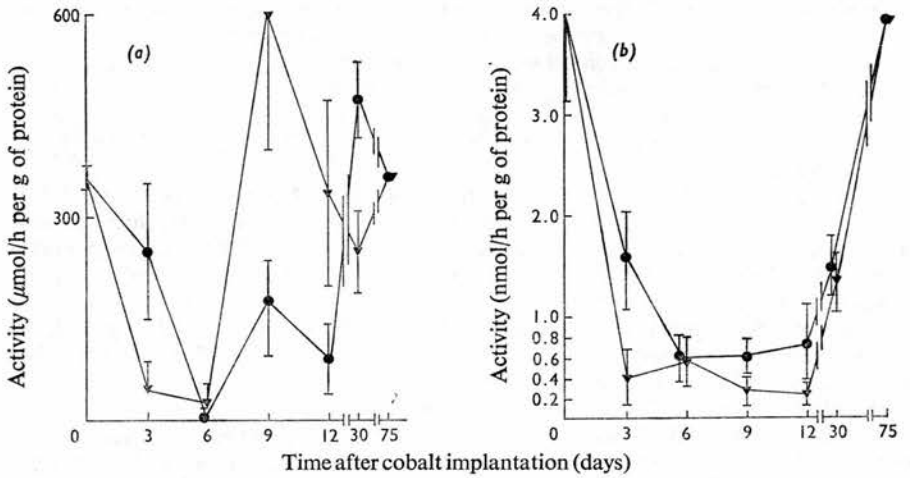

Fig. 1. *γ-Aminobutyrate transaminase* (*a*) *and tyrosine hydroxylase* (*b*) *activities in the frontal cortex of cobalt-treated rats*

▼, Primary focus; ●, secondary focus.

fibres from outside the cortex. The time-course for central-nervous-system regrowth has been found by many workers to be of the order of 50–100 days, for example, the re-innervation of the tongue after hypoglossal axotomy (Sumner & Sutherland, 1973). This correlates reasonably well with the rates of return to normal of the aminergic enzymes.

To extend these results, data on cortical and striatal concentrations of noradrenaline, dopamine and 5-hydroxytryptamine are being collected.

P. R. C. is a Medical Research Council research student, and P. C. E. is a Beit Memorial Research Fellow.

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The Effect of Neurotransmitter Release upon Phospholipid Composition and Fatty Acid Turnover in Synaptic Vesicles of *Torpedo marmorata* Electric Organ and Guinea-Pig Cerebral Cortex

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It has been reported that lysophosphatidylcholine can promote the fusion of erythrocytes and fibroblasts *in vitro* (Poole *et al.*, 1970) and, further, that high concentrations of this lysophosphoglyceride exist in chromaffin granules isolated from adrenal medulla (Blaschko *et al.*, 1967). Thus it has been proposed that this lysolipid may be involved in exocytosis, the mechanism whereby compounds stored in intracellular vesicles or granules are released to the extracellular medium (Poole *et al.*, 1970; Winkler *et al.*, 1974). Some support has been given to this proposal by the demonstration of phospholipase A activities in synaptic-vesicle fractions isolated from brain (Heilbronn, 1972; Gullis & Rowe, 1973). Yet a number of other types of vesicle have only low concentrations of lysophosphatidylcholine [as cited by Winkler *et al.* (1974)], and an enhanced turnover of [^{32}P]orthophosphate and [1-^{14}C]glycerol has not been demonstrated in lysophosphatidylcholine of adrenal-medulla slices stimulated by acetylcholine (Trifaró, 1969).

The present communication is based on a more detailed investigation of the possible involvement of lysophosphatidylcholine or other lysophosphoglycerides in synaptic vesicles during the release of neurotransmitters. For this purpose we have analysed synaptic vesicles from *Torpedo marmorata* electric organ and guinea-pig cerebral cortex.

In the first part of these investigations the electric organ was stimulated electrically *in vivo*, and crude synaptosomal fractions from cerebral cortex were incubated *in vitro* in the presence of 50mM-KCl, a treatment known to cause the release of transmitters (de Belleroche & Bradford, 1972). Synaptic vesicles were then isolated (Whittaker *et al.*, 1964, 1972) and their phospholipid compositions compared with those of controls. In further studies, crude synaptosomal fractions from cerebral cortex were incubated in the presence of labelled fatty acids and glucose and 50mM-KCl to determine if an enhanced turnover of radioactivity could be demonstrated in vesicles during neurotransmitter release.

The percentage compositions of the phospholipid fractions extracted from synaptic-vesicle preparations from electric organ and cerebral cortex are given in Table 1. It was found that electrical or 50mM-KCl stimulation did not significantly alter the phospholipid compositions of the vesicles. Lysophosphatidylcholine was the only lysophosphoglyceride demonstrable and its low concentration did not increase as a result of stimulation. Thus we conclude that there is no demonstrable generation of lysophosphoglyceride in synaptic vesicles as a result of neurotransmitter release.

Control experiments assessed the activities of enzyme markers in the vesicle fractions, the release of acetylcholine during stimulation and respiratory rates of the incubated samples.

To pursue the possibility of a rapidly turning over lysophosphoglyceride which is maintained at a very low concentration in synaptic vesicles, the labelling of vesicular phospholipid by radioactive fatty acids and radioactivity derived from glucose was studied by using the synaptosomal fraction from cerebral cortex. Generally, as measured by the specific radioactivities of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, the presence of 50mM-KCl in the incubation medium did not result in a significantly enhanced incorporation of [1-^{14}C]oleate, [1-^{14}C]arachidonate, [9,10-^{3}H]palmitate or radioactivity derived from [1-^{3}H]glucose into vesicular phospholipid. Thus we conclude that there is not an increased turnover of

Table 1. *Phospholipid compositions of synaptic vesicles and the effect of neurotransmitter release*

Results are expressed as percentages of total phospholipid phosphate and as means ± S.D. There were three separate determinations for each analysis. Guinea-pig synaptosomal fractions (P_2) were incubated with (Stimulated) and without (Unstimulated) KCl (50mM) for 15min *in vitro* after a 15min preincubation period. *Torpedo* electric organ was stimulated electrically *in vivo* (Stimulated) at 5/s for 30min through electrodes placed on the electric lobe (Zimmermann & Whittaker, 1974); a denervated organ served as control (Unstimulated).

	Percentage of total phospholipid			
	Torpedo vesicles		Guinea-pig vesicles	
	Unstimulated	Stimulated	Unstimulated	Stimulated
Lysophosphatidylcholine	0.4 ±0.3	0.3 ±0.2	0.6 ±0.2	0.5 ±0.1
Sphingomyelin	5.1 ±1.6	5.6 ±1.5	6.2 ±0.6	6.6 ±0.6
phosphatidylcholine	46.6 ±3.2	44.9 ±1.0	38.8 ±0.2	39.2 ±0.4
Phosphatidylinositol	5.1 ±1.1	4.6 ±0.3	3.8 ±0.3	3.5 ±0.3
Phosphatidylserine	12.6 ±2.3	11.5 ±0.7	12.8 ±0.8	12.6 ±1.2
Phosphatidylethanolamine	29.5 ±2.6	32.8 ±3.5	37.1 ±0.8	36.7 ±0.6
Phosphatidic acid	0.6 ±0.1	0.5 ±0.2	0.8 ±0.3	1.1 ±0.3
μmol of phospholipid phosphate/g of tissue	0.025 ± 0.006	0.018 ± 0.004	0.61 ± 0.15	0.58 ± 0.14

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Spread of Cobalt from a Cortical Epileptic Lesion Induced by a Cobalt-Gelatine Implant into the Frontal Cortex of the Rat

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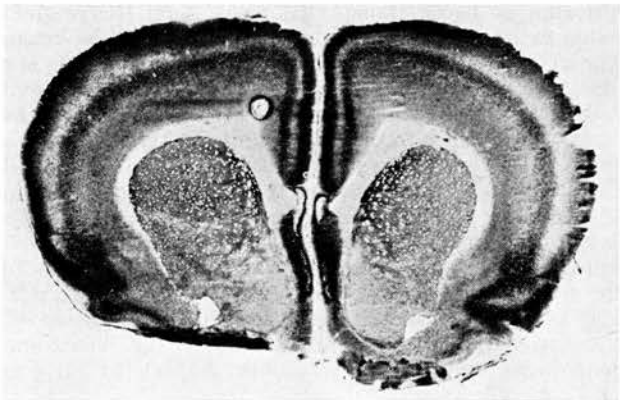
Summary. The spread of cobalt ions from cobalt induced epileptic foci in rats has been investigated. Atomic absorption spectrophotometry and heavy-metal histochemistry reveal cobalt ions spread very widely from the focus. Biochemical and physiological consequences for this model of epilepsy are discussed.

Kopeloff et al.[2] first reported that the application of powdered cobalt metal to the frontal cortex of the monkey produced epileptiform spikes in the electroencephalogram (EEG). Since Kopeloff's original observation the application of cobalt powder to the cortex or the insertion of cobalt gelatine pellets into brain has been used to produce reproducible epileptic foci in a variety of animals[3]. In the rat, application of cobalt to the cortex produces a distinct secondary focus in the contralateral cortex[4,5]. Because of the use of cobalt salts to trace axonal pathways[6] we were interested to know if the secondary focus, formed in the contralateral cortex of the rat did contain significant amounts of cobalt. If this were so then the value of this model would be reduced. Previous workers using this model[4,5] have suggested that the secondary focus in this model arises as a response to the spread of electrical signals from the primary focus across the corpus callosum and represents a response similar to the kindling phenomenon described by Goddard[7]. The presence of significant amounts of cobalt in the secondary focus would mean that it is probably solely caused by the presence of cobalt ions.

Cobalt-gelatine pellets prepared as described by Fischer et al.[8] of standard size 1 mm diameter and maximally 0.5 mm thick (representing at most 1 mg of cobalt metal in gelatine) were inserted into the right frontal cortex of male PVG rats as described in detail by Dow et al.[4]. The histochemical distribution of cobalt ions after this implant was investigated using the Timm[9] staining method at 4, 28 and 60 days after implantation. The rats were anaesthetized with an overdose of barbi-

A



A) The distribution of sulphide silver positive material (heavy metals) in the frontal cortex as revealed by the Timms method. Note the uneven distribution of staining in the cortical layers.

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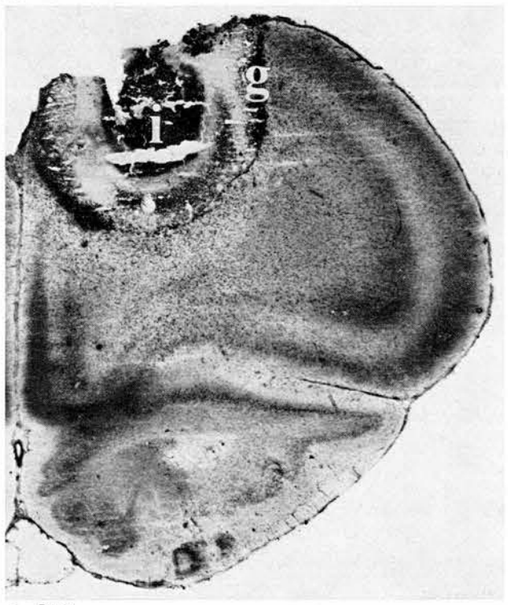
B

B) The effect of a cobalt-gelatine implant on the Timm-staining pattern of the frontal cortex. The rat was sacrificed 4 days after the operation to implant the cobalt. Note the marked increase in staining around the lesion, extending even in to the underlying caudate nucleus. In the secondary focus (contralateral cortex) the staining pattern seems more intense than in control sections incubated for the same time (Figure A). However it was not possible to be certain that there was definite increase in staining in the contralateral cortex.

C



C) A section through the centre of an established cobalt-epileptic focus (28 days survival). Note the definite calcified glial capsule surrounding the lesion, seeming to act as a barrier to further diffusion of cobalt. There is no longer any evidence of increased staining around the lesion and in contrast there is a decrease in Timm positive material outside the glial capsule.
Key to symbols on Figures B and C. d, area of cobalt diffusion; i, cobalt implant; g, glial capsule.

turate and perfused transcardially with phosphate buffered sulphide solution. Cryostat sections of the frozen brains were serially mounted and impregnated by physical development as described by Haug[10]. The results, of applying the Timm-staining method to the frontal cortex of rats 4 days after implantation revealed a marked increase in the density of histochemical staining around the cobalt-gelatine implant (Figure B) up to 2–3 mm in each direction from the original implant. Increased staining was evident in the cingulate cortex, corpus callosum and caudate nucleus below the lesion. We could demonstrate no unequivocal increase in staining in the contralateral cortex or secondary focus and the staining pattern in the contralateral cortex does not differ significantly from control sections (Figure B). The laminar pattern of stainable material agrees with previous observations[10] but the significance of the staining pattern is not understood. By 28 or 60 days (Figure C) the primary focus has become surrounded by a calcified glial capsule and no histochemical evidence of cobalt spread outside the glial capsule is evident. In fact by 28 days the glial capsule seems to provide a barrier to further spread of cobalt and this may account for the regression of the epileptic focus[2,11]. At no time during the development and regression of the cobalt focus could we be certain from histochemical staining that there was a spread of cobalt ions into the contralateral cortex, although we suspected this was occurring.

In order to further investigate the possible spread of cobalt we used atomic absorption spectrophotometry. This method would allow unequivocal demonstration of cobalt ions in the contralateral cortex if these were present. A Perkin-Elmer atomic absorptiometer, model HGA 360 was used and samples were read at a wavelength of 240 nm. Weighed pieces of brain were digested in concentrated HNO_3 and 20 μl of digest representing 1 mg of tissue was injected into the furnace. Using this program no loss of cobalt was detected during either the evaporation or ashing stages. No traces of cobalt were found in the reagents or incubation vessels. Known amounts of cobalt 1–50 ppm were taken through the procedure to provide a standard curve against which tissue data could be evaluated.

The results of these determinations are shown in the Table. Notice particularly the atomic absorption results show clearly that cobalt spreads extremely widely from the lesion site. At day 6 cobalt is found in significant amounts in the secondary focal area, in the occipital cortex both ipsi- and contralateral to the lesion site and also in the raphe nuclei some 1.5 cm from the cortical lesion site. The results are consistent with cobalt ions diffusing or being transported away from the original lesion to establish a gradient of cobalt through the brain. The amount of cobalt which spreads by diffusion is not likely to be very extensive outside the original lesion and it is probable that the majority of cobalt is spread to the rest of the brain by axonal transport. In agreement with this suggestion the levels of cobalt in the contralateral caudate follow closely the levels in the overlying frontal cortex with which it has connections. By day 21 the cobalt levels in the contralateral frontal cortex are significantly lower (1.8 $\pm$ 1.4 ppm) than the cobalt levels in the contralateral occipital cortex (5.3 $\pm$ 4.7 ppm) probably as a result of the extensive degeneration of neurones, axons and terminals in the ipsilateral frontal cortex and consequent reduction in transport. At day 21 there is still significant cobalt in the brain outside the implant. However by day 97 there is no longer detectable cobalt in brain areas outside the glial capsule.

[10] F.-M. S. Haug, Z. Anat. EntwGesch. *145*, 1 (1974).
[11] P. C. Emson and M. H. Joseph, Brain Res. *93*, 91 (1975).

Cobalt concentrations in ppm (dry matter) $\pm$ SD of the mean

	Cortex				Caudate nuclei		Raphe nuclei
	Frontal		Occipital				
	Lesion	Contralateral	Ipsi-	Contralateral	Ipsi-	Contralateral	
Day 6	>50 ppm	10.5$\pm$5.5	16.8$\pm$7.5	12.1$\pm$6.2	19.9$\pm$5.3	10.0$\pm$6.6	3.0$\pm$1.9
Day 21	>50 ppm	1.8$\pm$1.4	7.9$\pm$4.7	5.3$\pm$4.7	6.1$\pm$3.9	1.98$\pm$0.8	2.4$\pm$1.6
Day 97	No detectable cobalt						

There were 5 rats in each group. In all cases the glial capsule and any visible traces of cobalt-gelatine were removed from primary lesion samples. It was often not possible to remove all traces of the cobalt-gelatine, however, and therefore physiologically inactive cobalt may well have been assayed in the primary lesion. Excess values referred to represent values of over 50 ppm and were not quantified. Control rats and 97 day rats were assayed under the same conditions as the day 6 and 21 rats (see text) and under these condition no cobalt could be detected.

The implication of these results, for this and related models of epilepsy, are profound. Although the in vitro levels of cobalt (2–10 μM) which would be obtained in preparing a conventional brain homogenate (e.g. 1 mg brain tissue in 10 μl buffer) from an animal with a cobalt implant would not be sufficient to inhibit in vitro the enzymes we have previously studied (e.g. tyrosine hydroxylase choline acetyltransferase, glutamic acid decarboxylase)[12]. However, the levels in vivo (20–100 μM) will undoubtedly interfere with a wide range of metabolic process. In fact the wide spread of the cobalt ions and the possibilities of their concentration in different cellular compartments, such as nerve terminals suggests that a large area of the brain may become epileptogenic rather than just a small area around the original implant. These results indicate the need for caution in using heavy metal implants as models of epilepsy and suggest that the basis for cobalt induced epilepsy probably lies in a certain selectivity of cells, terminals and enzymes to the toxic effects of this ion in vivo. Certainly the secondary focus produced in this model of epilepsy although very interesting biochemically cannot be regarded as being untouched by the toxic effects of cobalt.

[12] P. R. Clayton and P. C. Emson, Biochem. Soc. Trans. *3*, 261 (1975).